

MOLECULAR AND IMMUNOLOGICAL ANALYSIS OF  
RECOMBINANT *BACTEROIDES FORSYTHUS* HEAT SHOCK  
PROTEIN 60

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## **PREFACE**

The work described in this thesis was carried out in the Molecular Biology laboratories of the Infection Research Group, Glasgow Dental School and Hospital, from October 1996 to September 1999 under the supervision of Marcello P. Riggio, BSc, PhD.

These studies represent original work carried out by the author, and have not been submitted in any form to any other university. Where use has been made of material provided by others, due acknowledgement has been made in the text.

October, 1999.

Helen Isobel Reid

## SUMMARY

This study addresses the putative role of *Bacteroides forsythus* heat shock protein 60 (hsp60) as a pathogenicity factor in periodontal disease by defining the molecular identity and antigenicity of the protein.

The gene encoding the heat shock protein 60 (hsp60) of *B. forsythus* was identified by PCR using degenerate hsp60 oligonucleotide primers, and by Southern blot hybridisation using probes derived from the *E. coli* hsp60 DNA sequence. The DNA sequence of the *B. forsythus* gene was determined, and was predicted to encode an amino acid of 544 amino acids with a molecular weight of 62kDa. The DNA and protein sequences displayed extremely high levels of conservation with hsp60 homologues from a range of other species, including those from other oral bacterial pathogens and human hsp60. The hsp60 gene of *Prevotella intermedia* was similarly identified, and the DNA sequence of the C terminal portion of the gene determined.

Bacterial hsp60 from a range of different species has been implicated in the pathogenic mechanisms of a wide range of diseases, and it is suggested that they may instigate autoimmune reactions in the host as a result of the high level of molecular conservation shared between bacterial and human hsp60 homologues. With the aim of evaluating the potential role of *B. forsythus* hsp60 in periodontitis, recombinant *B. forsythus* hsp60 was expressed in *E. coli* and its immunoreactivity determined by screening with 13 healthy adult and 11 chronic adult periodontitis serum samples. Both patient groups displayed a low level of IgG antibody reactivity with *B. forsythus* hsp60. The antibodies recognising the *B. forsythus* protein were cross-reactive with *E. coli* hsp60, and may have been initially raised against this species. These results indicate that

*B. forsythus* hsp60 is immunogenic, but do not suggest that this protein has an important role in the pathogenesis of adult periodontitis.

A *B. forsythus* genomic library was constructed in pUC18 and screened using a variety of techniques with the aim of identifying further immuno-reactive proteins of this species. Of the clones analysed, periodontitis patient serum did not display any reactivity. Random clones were selected for further analysis by DNA sequencing, and a clone carrying part of the *lac* operon of *B. forsythus* identified.

This thesis constitutes an original report on the molecular characterisation of part of the genome of the putative periodontal pathogen *Bacteroides forsythus*, and a preliminary investigation of host immune responses to a recombinant antigen of this species.

# **CHAPTER 1**

## **Introduction**

## ***Anatomy of the periodontium***

The term 'periodontium' describes the supporting tissues of the teeth whose main function is to dissipate forces applied to the dentition. These anatomical structures include the gingiva, periodontal ligament, cementum and alveolar bone. The gingiva is the oral mucosal lining which surrounds the teeth and covers the alveolar processes of the jaws. As well as withstanding masticatory forces, the gingiva functions as a protective seal around the teeth which prevents potentially pathogenic organisms from gaining internal access to the human body. Uniquely to the peripheral body defences (i.e. skin and gastrointestinal mucosa) that provide the continuous surface lining of the body, the gingiva is perforated (by teeth) which compromises its protective potential. Thus the tissues proximal to the teeth are appropriately designed to provide a tight seal around the teeth, and have developed repair and healing processes to cope with infections of the lining tissues (Schroeder and Listgarten, 1997; Jenkins and Allan, 1994). Cementum is specialised calcified tissue with collagen fibres, which forms the outermost covering of tooth roots. The periodontal ligament anchors in the root cementum and stretches to the alveolar bone that supports tooth sockets, linking and attaching teeth to bone. The main component of this connective tissue is collagen, but it also consists of several different cell populations (Beersten *et al*, 1997; Jenkins and Allan, 1994).

The healthy periodontium is characterised by a firm, pale pink gingiva that entirely fills the space between neighbouring teeth and does not bleed on probing. Alveolar bone is located 1mm short of the cemento-enamel junction

(Jenkins and Allan, 1994) and the gingiva is free from inflammation. However, as bacterial plaque is constantly accumulating at the gingival margin and can only be temporarily removed by oral hygiene measures, a limited amount of inflammatory exudate comprising of immune system components can be found in clinically healthy gingiva (Seymour *et al*, 1983).

### ***Periodontal disease***

Strictly speaking, periodontal disease describes a range of conditions affecting the various tissues which form the periodontium. Generally though, periodontal disease refers to periodontitis and gingivitis which are inflammatory and immune mediated reactions to microbial dental plaque. Gingivitis is the more common form of periodontal disease, with pathologic occurrences limited to the gingiva. The disease is clinically represented by the rubor and tumor characteristic of any inflammatory response and the gingival tissue is likely to bleed upon gentle probing (Taichman and Lindhe, 1989). Periodontitis is a far more invasive disease with alveolar bone, periodontal ligament and root cementum affected by the disease processes. Clinically, periodontitis is manifested by the formation of open pockets around the teeth and advanced cases result in alveolar bone resorption resulting in loosening, and loss of, teeth (for a more detailed description of clinical features see Kinane and Lindhe, 1998). The World Workshop in Clinical Periodontics (1989) classified different forms of periodontitis as listed:

- 1) Adult periodontitis
- 2) Early onset periodontitis: i) prepubertal periodontitis (localised and generalised)



ii) juvenile periodontitis (localised and generalised)

iii) rapidly progressive periodontitis

3) Periodontitis associated with systemic disease

4) Necrotising ulcerative periodontitis

5) Refractory periodontitis

The differences between the age-of-onset groups are self evident, although there also appears to be variances in the microbial complexes that are associated with these diseases (Genco *et al*, 1988). Adult periodontitis is the most common form of periodontitis. Cases of periodontitis which are associated with systemic disease are thought to be symptoms attributable to an underlying disease or disorder which might predispose the individual to disease. Necrotising ulcerative periodontitis describes a rapidly progressive condition that may result in severely advanced periodontitis, and is associated with spirochaetal populations in microbial plaque. Refractory periodontitis refers to a disease type that does not respond to treatment that is effective against other forms of the disease.

### ***Pathogenesis of periodontitis***

Current research implicates plaque associated micro-organisms in provoking inflammatory and immune responses that may lead to periodontal disease. Whilst bacteria may exhibit factors that are directly pathogenic, such as the expression of toxins, it is commonly held that it is unchecked host immune and inflammatory processes, initially raised as a protective response against bacteria, that are accountable for the injury to tissues seen in periodontal disease. As only a small subset of the periodontitis population develop

advanced periodontal destruction (Jenkins and Kinane, 1989; Loe *et al*, 1986) periodontal disease is thought to be subject related, with those few individuals who experience extensive destruction of the periodontium thought to have a localised imbalance in the host response to micro-organisms that renders them susceptible to disease (Kinane, 1997). It is not as yet clearly understood why some patients will develop advanced disease affecting several teeth whilst others will suffer only from mild lesions at the gingiva which do not affect connective tissue or supporting bone. However a number of host factors, such as age and smoking and drinking habits, are known to increase the likelihood of a person developing periodontal disease. Host factors alone though do not cause periodontal disease; the root cause, undeniably, is the mixed microbial population thriving at the gingival margin which constitutes dental plaque.

### ***Aetiology of periodontal disease***

#### ***i) Problems***

Attempts to determine the aetiological agents of periodontal disease have been hindered by a number of problems. The term 'periodontal disease' encompasses a range of clinically different diseases such that patients presenting with similar symptoms might not necessarily be suffering from the same microbial infection, and this makes classification of disease types and status difficult. Although periodontal disease is regarded as a microbial infection of discrete sites of the periodontium, a range of host factors also significantly contribute to disease progression. Thus the aetiology of periodontitis is attributed to a combination of both microbial and host factors.

### ***ii) Microbial factors***

Data evidencing a primary role for bacteria causing periodontal disease is virtually conclusive. Reports to support this microbial paradigm come from a large number of studies demonstrating:

- i) a strong association between the amount of bacterial plaque and the severity of periodontitis and level of bone loss
- ii) rigorous plaque control procedures, such as mechanical removal of plaque, can successfully treat disease
- iii) the success of antibiotics targeted at microbial plaque at controlling different forms of periodontal infections.

Problems arise when attempts are made to define which of the 400 species that may be present in periodontal pockets are pathogenic. Further difficulties are imposed by the inability to culture some species of bacteria in the laboratory, the difficulty in identifying which pathogens are secondary opportunistic invaders, and determining which strains of specific species are the most virulent. As mentioned later, strong evidence exists to indicate mixed infections as crucial in the etiology of the disease. This further impedes the microbiologist's ability to pin-point pathogenic species as all the potential bacterial pairs or mixed populations have to be considered.

### ***iii) Host factors***

Although micro-organisms are necessary for periodontal disease to occur, it appears that they alone are not sufficient to cause disease. Several host factors make an important contribution to the disease process. These include the genotype of the host. Some genotypes, by means of family studies, have been

noted to be particularly susceptible to periodontal diseases (Genco, 1996).

Other risk factors that affect the likelihood of an individual developing these oral afflictions include gender, age, stress, diabetes mellitus and cigarette smoking (Genco, 1996; Zambon *et al*, 1996a).

### ***The role of bacteria in periodontal disease***

Specific aetiological agents of periodontal infections have been sought for over a century. Opinion on what these causative agents may be, and their role in disease pathogenesis, has vacillated during this period.

#### ***i) Non specific plaque hypothesis***

From the mid 1920s until the 1960s, little work was done to identify species of bacteria involved in periodontal infections. Generally, bacteria were thought to play some role in the pathogenic process, and the need for plaque control to prevent and treat disease was recognised. However, no specific microbial organisms were targeted as causative agents. This adheres to the 'non specific plaque hypothesis', which holds that any sufficiently large population of micro-organisms at the gingival margin could provoke an inflammatory reaction, irrespective of the microbial composition of the plaque. According to this hypothesis, bacteria are deemed to act only as triggers of an inflammatory response that would eventually lead to the destruction of the periodontium (Socransky and Haffajee, 1992).

#### ***ii) Specific plaque hypothesis***

Since the 1960s, opinion on the possible cause of periodontal infections has swayed once again, and now favours the 'specific plaque hypothesis', a

supposition that had once been held at the beginning of the century also. The main tenet of this hypothesis is that specific micro-organisms relate to the aetiology of different periodontal diseases. Supporting this argument for an intrinsically pathogenic role for specific species are studies that attempt to elucidate the composition of bacterial plaque in periodontal infections.

Differences in the microbial population have been found in varying forms of periodontal disease, and also major distinctions between the microbiota of healthy sites as compared to diseased ones have been noted.

Distinct problems are encountered when attempts are made to identify and define specific periodontal pathogens. Some of these are technical difficulties; obtaining samples from the correct periodontal site without contamination from other microbial populations, the need to perhaps keep the sample in a strictly anaerobic environment, the inability to culture all species present. Should a sample be successfully cultured, the even more daunting prospect of recognising a pathogenic species, which may also be part of the normal microbial flora, from the approximately 400 other species in subgingival plaque yet remains.

Despite these hindrances, current consensus recognises a number of species as periodontal pathogens, playing a crucial role in the course of the disease. This list includes *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Bacteroides forsythus*, *Prevotella intermedia* and *Fusobacterium nucleatum* as the main suspects, but includes many less studied species as well.

### ***Pathogenic synergism***

Bacteria isolated from dental plaque are always part of a mixed and variant microbial population. This can incur advantages to an organism that requires prior colonisation of a site by another species before it can establish itself.

Alternatively, a species possessing antibiotic resistance or the ability to withstand the battery of host immune defences can shield a more vulnerable pathogen from attack.

### ***Periodontopathogens***

#### *i) Actinobacillus actinomycetemcomitans*

*Actinobacillus actinomycetemcomitans* is a non-motile, Gram negative saccharolytic rod. Its strong association with periodontal disease is reflected in the large numbers of publications that implicate this species as an aetiological agent of various forms of periodontal disease (reviewed by Haffajee and Socransky, 1994). The bacterium appears to be most strongly associated with juvenile forms of periodontal disease, as evidenced by a marked increase in frequency of detection and higher numbers of the bacterium in lesions from LJP patients as compared to samples from inactive sites or from healthy controls (Haffajee *et al*, 1984; Mandell *et al*, 1981; Tanner *et al*, 1984; Zambon *et al*, 1996b). *A. actinomycetemcomitans* is isolated less frequently in samples from adult periodontitis patients in which other putative pathogens seem to dominate (Rodenburg *et al*, 1990).

Elevated antibody levels to *A. actinomycetemcomitans* in both localized juvenile and some adult periodontitis serum and saliva samples also suggest that *A. actinomycetemcomitans* plays a pathogenic role in various types of periodontitis (Ebersole *et al*, 1987; Ebersole *et al*, 1991; Haffajee and Socransky, 1994).

### ii) *Porphyromonas gingivalis*

Another microorganism frequently cited as a likely periodontal pathogen is *Porphyromonas gingivalis*. *P. gingivalis* is a Gram negative, anaerobic asaccharolytic non-motile bacterium that forms black colonies when grown on blood agar. *P. gingivalis* is a member of the *Bacteroidaceae* group of microorganisms, and was previously classified as *Bacteroides gingivalis* (Haffajee and Socransky, 1994). *P. gingivalis* has been associated with various forms of periodontitis, as assessed by bacterial numbers and frequency of detection (Choi *et al*, 1990; Haffajee *et al*, 1988a; Slots *et al*, 1986; White and Mayrand, 1981). However, as is the case with all other putative periodontal pathogens, patients with periodontitis frequently present who are not infected with this organism (Moore and Moore, 1994). A host antibody response to *P. gingivalis* in periodontitis patients has been widely demonstrated (Haffajee and Socransky, 1994).

The organism is also a focus of interest due to the high number of virulence factors it appears to possess. These include an IgG degrading protease (Grenier *et al*, 1989), collagenase (Uitto *et al*, 1988) and bone resorbing factors (Bom-van Noorloos *et al*, 1989).

### iii) *Prevotella intermedia*

*Prevotella intermedia*, similar to *P. gingivalis*, is a black pigmented anaerobe of the *Bacteroides* group. Its name and classification derive from the fact that within the *Bacteroides* group, species were identified which were neither asaccharolytic (such as *P. gingivalis*) or highly saccharolytic (such as *Prevotella melaninogenica*), but fermented carbohydrates at an 'intermediate' level (Haffajee and Socransky, 1994).

Elevated levels of *P. intermedia* have been demonstrated in various forms of periodontitis (Dzink *et al*, 1985; Dzink *et al*, 1988; Socransky and Haffajee, 1992), notably so in lesions of acute necrotizing ulcerative gingivitis (Loesche *et al*, 1982). Serum antibody titres to this species are raised in some subjects (Haffajee *et al*, 1988b).

#### *iv) Fusobacterium nucleatum*

Another Gram negative anaerobic rod, *F. nucleatum* is a well recognised frequent pathogen of other sites in the human body, and is recovered from infectious abscesses of the brain and other vital organs (Moore and Moore, 1994). *F. nucleatum* is the most frequently isolated species in subgingival plaque samples (Dzink *et al*, 1985; Dzink *et al*, 1988). It is invariably detected in sites with low gingival disease scores, which indicates that this species is a major role player in the initiation of periodontal disease (Moore and Moore, 1994). *F. nucleatum* is proposed to be part of a microbial complex of species that are commonly isolated in association with each other. It appears to be necessary for the *F. nucleatum* complex to be present at a periodontal site before other species can become established (Socransky *et al*, 1988), and this supports the idea that *F. nucleatum* is a primary coloniser of periodontally diseased sites.

#### *v) Other species*

A range of other microbial species have been implicated as periodontal pathogens. Many of these have only been recently characterised or new subspecies have been differentiated after molecular taxonomic studies such as 16S ribosomal RNA sequencing. Due to this, data to suggest their role as aetiologic agents of periodontal disease are more limited than they are for the bacteria previously mentioned. These



lesser-studied species include *Eikenella corrodens*, *Campylobacter rectus*, *Wolinella recta*, *Peptostreptococcus micros* and *Eubacterium* and *Selenomonas* species (Haffajee and Socransky, 1994; Socransky *et al*, 1998; Tanner *et al*, 1987a). It is likely that ongoing work to evaluate their pathogenic role will substantiate preliminary data that finds these species more frequently and in higher numbers in active disease sites of patients as compared to healthy sites.

### ***Bacteroides forsythus***

*Bacteroides forsythus* is a strictly anaerobic Gram negative bacillus. Tanner *et al* first described it as a 'fusiform' *Bacteroides* in 1979, when it was isolated from patients with advancing periodontitis. The bacterium fitted descriptions for *Fusobacterium nucleatum*, but differences in growth requirements, dye and antibiotic sensitivities led the group to believe that the bacterium was a new species. Tanner went on to describe the new species, *Bacteroides forsythus*, in 1986 (Tanner *et al*, 1986).

### ***Identification of B. forsythus***

#### ***i) Culture techniques***

*B. forsythus* is a slow growing organism, and produces very small colonies that are only detectable by culture after 7-14 days incubation. The bacterium has fastidious growth requirements, requiring the addition of N-acetyl muramic acid to the culture medium to enhance growth (Wyss, 1989). For these reasons the bacterium is difficult to isolate by culture and has perhaps been under represented in studies attempting to identify and enumerate aetiologic agents of oral infections by standard culture methods alone.

In studies from an era prior to molecular and immunological detection methods, which depended on less sensitive culture techniques, *B. forsythus* or 'fusiform' *Bacteroides* was isolated from active destructive periodontal lesions but was not found at inactive periodontitis sites (inactive sites had comparable pocket depth to active sites, but the attachment level did not alter during the monitoring period) (Dzink *et al*, 1985). Fusiform *Bacteroides* were detected in some samples from advancing periodontitis sites (Tanner *et al*, 1979), but as the species had not been classified at this stage it is likely that these figures are inaccurate.

Although the organism was detected more frequently and in higher numbers at sites of active and destructive periodontitis lesions as compared to inactive and healthy sites (Dzink *et al*, 1985; Lai *et al*, 1987), initial indications were that *B. forsythus* was a fairly uncommon inhabitant of the diseased periodontium. From 119 lesions studied, Dzink *et al* (1985) detected only 8 *B. forsythus* positive samples. Socransky *et al* (1988) studying a different group of patients, found only 10% of sites sampled to be *B. forsythus* positive.

Despite the apparently low prevalence of the organism, interest in the species persisted as up until this time *B. forsythus* was only associated with active periodontitis sites. This strongly indicated a role for the bacterium in the pathogenesis of active destructive periodontal lesions.

## **ii) Serological techniques**

The development of molecular and immunological techniques replace culture techniques for the assessment of the prevalence of *B. forsythus* in clinical samples. These new methodologies include the use of serological agents, for

example monoclonal antibodies, whose specificity for a given species can be utilised to indirectly detect micro-organisms.

Several groups have utilised immunological techniques such as the enzyme linked immuno-sorbent assay (ELISA) and indirect immunofluorescence microscopy to identify *B. forsythus* in various periodontitis samples (Califano *et al*, 1992; Di Murro *et al*, 1997; Gmur *et al*, 1989; Lai *et al*, 1987; Tanner *et al*, 1998; Wernerfelmayr *et al*, 1988).

Indirect immunofluorescence microscopy performed soon after the new *B. forsythus* species had been characterised extended earlier observations and showed *B. forsythus* to have a strong positive association with subgingival plaque of severe gingivitis and untreated adult periodontitis as well as recurrent periodontitis (Lai *et al*, 1987). Few healthy subjects appeared to be infected with the species at this time.

### **iii) Molecular techniques**

Of greater sensitivity, with the ability to detect microbial DNA from as few as  $10^3$  cells, are the molecular techniques of DNA hybridisation (Southern and dot blotting) and the polymerase chain reaction (PCR). The use of PCR (Goncalves and Mouton, 1998; Meurman *et al*, 1997) and DNA hybridisation (Lai *et al*, 1987; Socransky *et al*, 1998; Tanner *et al*, 1998) for identifying periodontal bacteria in clinical samples is well documented.

The advent of molecular DNA detection methods for identifying *B. forsythus* has been recent enough to mean that data describing results from molecular studies constitutes the basis of many current publications. Genomic probes allow direct detection of the species, and so far have identified *B. forsythus* in 73% of 614

subjects with deep periodontal pockets (Lotufo *et al*, 1994). Studying a prodigious number of samples, Listgarten *et al* (1995) identified 84% of 993 plaque samples to be positive for *B. forsythus*.

For such a fastidious organism that requires a supplemented growth medium and anaerobic sampling and culturing to be successfully isolated, molecular and immunological detection methods appear to perform far better than culture techniques. In a study comparing culture and molecular methods for their ability to detect microbial anaerobic periodontal pathogens, Loesche *et al* (1992) found the prevalence of *P. gingivalis* and *B. forsythus* to be 89% and 64% respectively, as assessed by ELISA. Culture of the same plaque samples identified *P. gingivalis* in only 43%, and *B. forsythus* in 25% of the samples. Improved detection methods have resulted in more recent reports highlighting the realisation that *B. forsythus* is a far more common constituent of diseased and healthy periodontal flora than was previously held to be the case. The technique of counting fluorescent immunologically labelled cells by aid of microscopy was applied to a group of patients who maintained a high level of oral hygiene, and were deemed unlikely to suffer from active periodontal destruction. *B. forsythus* was found to be present in up to 50% of sites sampled over a 12 month period. The organism was isolated from shallow gingival pockets, unaffected by attachment loss or alveolar bone loss (Gmür *et al*, 1989). Thus the opinion that the mere finding of *B. forsythus* in a periodontal pocket may be suggestive of progressing disease could no longer hold. This fact was consolidated by evidence that showed *B. forsythus* could be isolated from the supragingival plaque of subjects with excellent oral health and hygiene

(Gmür and Guggenheim, 1994). However, Gmür's study showed that *B. forsythus* colonisation levels increased proportionally with the probing depth of periodontal lesions (Gmür *et al*, 1989).

The most recent studies find *B. forsythus* to be a major species in active periodontitis lesions, and also find it present at lower levels in healthy sites. Surprisingly, some of this data is still obtained via culture techniques (Tanner *et al*, 1998) despite the fact that the general consensus of opinion seems to deem this method as lacking sufficient sensitivity for accurate reports.

### ***Mutual association of B. forsythus with other species***

It has been ascertained that *B. forsythus* frequently occurs in periodontitis associated with other species of bacteria. A number of studies report *P. gingivalis* to be commonly identified with *B. forsythus* in periodontitis. Lotufo *et al* found concurrently growing *B. forsythus* and *P. gingivalis* in 55% of patients sampled (Lotufo *et al*, 1994). Listgarten's study of 993 plaque samples from periodontitis sites similarly found 84% to be *B. forsythus* positive and 63% *P. gingivalis* positive (Listgarten *et al*, 1995).

Other studies have found *B. forsythus* to be co-isolated from infected sites with *F. nucleatum*. Tanner *et al* found 12 out of 13 *B. forsythus* positive sites were colonized by *F. nucleatum*, and it has been demonstrated that *F. nucleatum* boosts the growth of *B. forsythus* in culture (Tanner *et al*, 1986).

Using DNA probes to identify positive sites, Rams *et al* (1996) sampled 329 moderate-severe periodontitis patients. 77.5% of the patients they studied had sites infected by *P. gingivalis* and 76.3% were positive for *B. forsythus*, establishing a significant joint association in the occurrence of the two species.

Socransky *et al* (1998) also used genomic DNA probes to determine the prevalence of and associations between 40 subgingival species in 13,261 plaque samples collected from 185 subjects. Data was subjected to statistical analysis (multiple cluster and community ordination analyses). Results revealed that the putative periodontal pathogens formed 5 major microbial complexes, and that species consistently formed the same complexes irrespective of the analytical method employed. One complex consisted of *B. forsythus*, *P. gingivalis* and *Treponema denticola*. Although isolated alone from a proportion of sites, *B. forsythus* was more frequently identified at sites also inhabited by *P. gingivalis* and *T. denticola*. *B. forsythus* was more likely to be detected in the absence of *P. gingivalis* than *P. gingivalis* was to be found without *B. forsythus*. Members of this complex were found more commonly and in higher numbers in deeper periodontal pockets, and their prevalence also showed a marked relation to bleeding on probing. A second microbial complex contained, amongst other species, *F. nucleatum*. The data indicated that members of the first complex (i.e. *B. forsythus*, *P. gingivalis* and *T. denticola*) were rarely found in the absence of members of the *F. nucleatum* complex. However the *F. nucleatum* complex could be found without members of the first complex being present. This would suggest that the *F. nucleatum* complex pre-colonises sites prior to the *B. forsythus* complex. These findings offer a feasible explanation for earlier reports of *B. forsythus* being associated in infected periodontal sites with both *P. gingivalis* and *F. nucleatum*.

### ***Evidence implicating B. forsythus in periodontal disease***

Since Koch described a microbial etiologic agent of tuberculosis in 1882, 'Koch's postulates' have been applied as the dogma for identifying a causative agent of any infection. According to his postulates, a link between an infectious agent and disease can be proven when

- i) the causal agent can be isolated from every case of the disease
- ii) the agent is not identified in other forms of the disease, or non pathogenically
- iii) pure cultures of the organism *in vitro* can induce disease when experimental animals are infected with it *in vivo*.

The high number of species implicated in the aetiology of periodontal disease, some of which may be part of the normal periodontal flora, along with the fact that a good animal model of periodontal disease is still lacking, means that currently Koch's postulates would not serve well to identify the infectious agent of periodontal disease. In an attempt to overcome these difficulties, Socransky proposed alternative criteria to use when identifying periodontal pathogens (Socransky, 1977). As applied to *B. forsythus*, these are as follows:

- i) *The putative pathogen is present in high numbers in disease, but low numbers in health.*

A considerable number of reviews looking at microbiological samples have shown that the number of bacterial cells present in the plaque of the healthy gingiva is low, with Gram positive species predominating. With the onset of periodontal disease, bacterial numbers increase and the population shifts to a predominately Gram negative one (Moore and Moore, 1994).

Studies showing that levels of *B. forsythus* increase in patients with periodontal disease have been well documented (Dzink *et al*, 1988; Gmur *et al*, 1989; Lai *et al*, 1987; Lotufo *et al*, 1994; Tanner *et al*, 1979; Tanner *et al*, 1998).

ii) *Patients infected with the suspected pathogen develop high antibody titers in serum, saliva and gingival crevicular fluid.*

Ebersole *et al* (1987) presented evidence that elevated serum antibody responses show a close association with the species eliciting the periodontal infection in question. A review of the literature that indicts some of the periodontal pathogens in such a manner can be viewed in Ebersole and Taubman, (1994).

The first reports measuring the serum concentration and frequency of seropositivity for IgG antibodies reactive with *B. forsythus* in periodontitis patients have been published recently. Gu *et al* (1997), report that patients with rapidly progressing periodontitis do not mount an antibody response to the antigens of *B. forsythus*, although antibody titers to other periodontal pathogens in these patients were elevated. Similarly, Califano and colleagues have reported that levels of IgG antibody to *B. forsythus* do not increase significantly in periodontitis patients, and that the percentage of patients seropositive for *B. forsythus* was the same in both periodontitis and non-periodontitis patients (Califano *et al*, 1992).

The absence of a strong antibody response to *B. forsythus* did not indicate an absence of *B. forsythus* in the periodontal microbiota (Gu *et al*, 1997). The lack of a humoral antibody response to the pathogen could be explained if the organism had poor 'invasive potential', thus confining the antigens to the



periodontal pocket. Alternatively, the organism may have the ability to invade host cells, but the antigens it expresses are not good immunogens.

It is possible the *B. forsythus* can invade host cells, yet manages to escape antibody recognition via an as yet unknown evasion mechanism. Should this be the case, the organism would have impressive pathogenic potential, and would be a good risk factor for disease.

*iii) The micro-organism can demonstrate in vivo production of virulence factors that can be correlated with disease.*

*B. forsythus* produces a trypsin-like enzyme (Tanner *et al*, 1985) and a sialidase (Holst and Bramanti, 1991), both of which may be virulence factors that have a pathogenic role in periodontal disease. The organism has also been shown to express a surface layer (S-layer) of crystalline protein, formed from serrated subunits (Kerosuo, 1988). It is thought the S-layer may function to strengthen the bacterial cell wall, and speculatively, may also contribute to the virulence of the organism as other oral bacteria possessing an S-layer have been isolated from acute oral infections.

*B. forsythus* has been shown to be capable of invasion; a small number (~1%) of epithelial cells harvested from periodontal pockets were found to contain *B. forsythus* cells (Dibart *et al*, 1995).

*iv) Experimental implantation of the organism into an animal model should result in expression of some of the characteristics of the disease.*

Several animal models for periodontitis currently exist, these include systems in rodents, dogs and non-human primates (Haffajee and Socransky, 1994). It

should be noted that problems exist with animal models of periodontitis: periodontal disease does not spontaneously occur in many species of animals, or only becomes established in very old animals. Differences between the indigenous oral micro-flora of humans and laboratory reared experimental animals may affect the interpretation of some studies. Also, bacterial species may have a preference for specific adherence factors on epithelial tissue or on other oral bacteria. It is likely that these ligands necessary for successful attachment of an invading organism vary significantly between genera. Studies in monkeys have shown that numbers of Gram negative anaerobes, including *Bacteroides* species, increase at sites of periodontal loss in animals (Kornman *et al*, 1981; Slots and Hausmann, 1979). An analysis of the subgingival micro-flora of 16 *Macaca nemestrina* monkeys with naturally occurring gingivitis revealed that all the animals harboured *B. forsythus*, along with *P. gingivalis* and *F. nucleatum*. *A. actinomycetemcomitans* was only found in the younger animals within the group studied (Persson *et al*, 1993).

v) *Treatment that removes the suspected pathogen from the infected sites results in clinical improvement.*

Treatment of periodontitis involves destruction of the infectious subgingival microbiota, either via antibiotic therapy or by mechanical means such as subgingival scaling. Whilst both methods can be extremely efficacious, their methods of attack are not targeted at specific micro-organisms, but at the whole microbial plaque population. Disruption of the structure of the polluting biofilm, that often requires close physical proximity between cells for successful symbiosis, may be sufficient to terminate disease.

In a study of patients with refractory periodontitis, a group of subjects who did not appear to respond to therapy were found to be infected with *B. forsythus* (Haffajee *et al*, 1986). In these cases, it is possible that the patient's 'non-specific' treatment was not sufficient to remove *B. forsythus*, and led to a worsening of their condition. Thus there is a cause for the development of species-specific therapies for the treatment of some cases of periodontitis.

In conclusion, that data available presents convincing evidence that *B. forsythus* is a periodontal pathogen. A brief perusal of current papers on the microbiology of periodontitis highlights the diversity of micro-organisms implicated in the disease, and several differing reports on the microbial flora have been reported. These varying reports may be due to differences in sampling sites, sampling techniques and methods used to identify isolates.

*B. forsythus* may be present as part of a symbiotic relationship, as indicated by reports on its mutual association with other species. It may also be an opportunistic pathogen, only becoming established after the disease process has been initiated.

### ***Heat shock proteins***

The heat shock proteins represent a large group of proteins, within which individual heat shock protein (hsp) families are defined by their apparent molecular weight. The main families of hsp are hsp90, hsp70, hsp60 and small hsps. The different families all have in common the ability to act as a chaperone to other cellular proteins. This functional conservation is reflected at the

genomic level, with a measure of sequence conservation maintained throughout the group. The families differ with regards to their binding specificities. For example, the hsp90 proteins have an affinity for steroid receptors, and bind to them to prevent untimely interactions between the steroid receptors and DNA. Hsp70 proteins are known to be involved with protein translocation and the assembly of immunoglobulins. Hsp60 predominantly functions to fold and unfold polypeptides to ensure the correct assemblage of oligomeric protein complexes and to maintain proteins to be transported in a format compatible with translocation (see reviews in Kaufmann, 1990; Ellis and Vandervies, 1991). As chaperones, each family of proteins acts to prevent incorrect protein interactions, or the formation of protein aggregates. None of the chaperones possess the steric information that their charges require to fold or interact correctly, nor are the chaperones incorporated into the final protein structures and complexes, but dissociate when the time or place is appropriate.

### ***Heat shock protein 60***

The term 'molecular chaperone' was first used to describe a protein identified in *Xenopus* eggs that bound histones and transferred them to DNA to form nucleosomes. The binding of the chaperone prevented incorrect interactions between histones and DNA occurring, and allowed only selected interactions to take place (Laskey *et al*, 1978). After this time, proteins found to share a similar role in the assembly of other multi-protein complexes were identified in *E. coli* and in plant chloroplasts. The groE protein of *E. coli* was found to be essential for the correct assembly of T4 bacteriophage in the bacterium (Hendrix, 1979), whilst a chloroplast protein was found to be necessary for the assembly from

sub-units of the photosynthetic CO<sub>2</sub>-fixing enzyme, ribulose biphosphate carboxylase-oxygenase (Barracclough and Ellis, 1980). Thereafter, Ellis proposed that these proteins from seemingly disparate organisms belonged to a class of cellular proteins, whose common function was to aid the correct folding and formation of polypeptide chains and multimeric structures (Ellis, 1987). The protein family was the hsp60 group of chaperones, also referred to as chaperonins and groEL-like proteins. The hsp60 proteins are thought to function by maintaining cellular proteins in an unfolded conformation, until such time that they are released from the chaperone and are able to undergo intra- or inter-molecular folding. By maintaining proteins in an unfolded conformation, hsp60 is also able to aid protein translocation and export (Zeilstra-Ryalls *et al*, 1991). Hsp60 also possesses the ability to unfold a protein or protein complex that has already adopted a tertiary structure, such that hsp60 can cause incorrectly formed protein aggregates to dissociate, and can cause a ready folded protein to assume a conformation more amenable for export. The variety of protein substrates that hsp60 can bind is known to be very large, but is not fully defined (Zeilstra-Ryalls *et al*, 1991).

Cellular stress, such as heat shock, typically causes protein to denature. Hsp60 can therefore protect cells from the adverse effects of stress by reducing the amount of free, denatured protein in the cell, and aiding the re-formation of functional protein structures. Many oligomeric structures in cells are required for essential biological functions to occur, such as the biosynthesis of amino acids, RNA synthesis, DNA replication and protein translocation. As these structures would not be able to form without the aid of hsp60, these molecular chaperones are essential for cell viability even in non-hostile conditions (Craig *et al*, 1993).

### ***Hsp60 expression and regulation***

Heat shock proteins are amongst the most abundant proteins in the biosphere (Kaufmann, 1990). Constitutively expressed, they account for up to 5% of all proteins present in bacteria under normal conditions, and up to 40% of total protein expressed following cellular stress (Kaufmann *et al*, 1991; DeBruyn *et al*, 1987). The term 'heat shock' protein is something of a misnomer, as various cellular insults induce the increased expression of hsp60 proteins. These stresses include pH decrease, exposure to reactive oxygen metabolites and starvation (Buchmeier and Heffron, 1990).

The level of hsp60 expression within a cell is thought to be determined by a feedback loop that indicates the equilibria in the cell between folded and unfolded proteins, and between protein-associated and free hsp60 molecules. Following cell stress, the level of unfolded, denatured proteins in the cell increases, and the amount of free hsp60 decreases correspondingly. This decrease in free hsp60 is thought to enable a heat shock transcription factor to bind to a heat shock gene promoter, thus causing an increase in the expression of the gene product such that the level of free hsp60 will rise again within the cell. When the free hsp60 level is adequate, the heat shock transcription factor is bound by hsp60 and is unable to induce high levels of hsp60 synthesis (Kaufmann, 1994).

### ***Hsp60 and disease***

A prodigious and ever increasing number of observations implicate hsp60

involvement in a wide range of clinical situations. These range from bacterial infections where the hsp60 is a common antigen expressed by bacterial species, and flags potential pathogens for rapid attack by the host immune response (Kaufmann, 1991; Kaufmann *et al*, 1991; Kaufmann, 1992b; Shinnick, 1991), to organ specific inflammatory diseases, such as arthritis (Boog *et al*, 1992; DeGraeff Meeder *et al*, 1991; Gaston *et al*, 1990), peptic ulcer disease (Amini *et al*, 1996; Yamaguchi *et al*, 1997), chlamydia (Horner *et al*, 1996; Morrison *et al*, 1989; Taylor *et al*, 1990), kidney disease (Warr *et al*, 1997), heart and vascular disease (De Stefano *et al*, 1993; Desvarieux *et al*, 1999; Hopplicher *et al*, 1996; Matilla *et al*, 1989; Wu *et al*, 1999) and a number of autoimmune disorders (Cohen, 1992; Georgopoulos and McFarland, 1989; Jones *et al*, 1993; Kaufmann, 1994). More recently the potential role of hsp60 in diseases of the nervous system, such as myasthenia gravis (Astarloa *et al*, 1996), Alzheimer's disease (Hamos *et al*, 1991) and multiple sclerosis (Raine *et al*, 1996), and in cancer (Hsu and Hsu *et al*, 1998; Ito *et al*, 1998) has been studied. Each study confirms the highly antigenic nature of hsp60. Of particular relevance to this study are the hsp60 studies undertaken by others with regard to inflammatory diseases and autoimmune diseases. The most extensive work by far has been done on arthritis. Interestingly, arthritis and periodontitis share a number of similarities. Both are chronic inflammatory diseases, and result in bone resorption, possibly as a result of autoimmune reactions. Thus the literature on hsp60 and arthritis will be studied more closely below. Literature on hsp60 and autoimmune disease will also be examined.

### ***Hsp60 and arthritis***

The pathogenic role of the 65 kd hsp of *M. tuberculosis* (mycobacterial 65-kD hsp, Mb hsp65; an hsp60 homologue) in arthritis has been widely studied. In humans, both cellular and humoral reactions to Mb hsp65 have been reported in rheumatoid arthritis (RA) and juvenile chronic arthritis (JCA) patients (Boog *et al*, 1992; DeGraeff-Meeder *et al*, 1990; Haregewoin *et al*, 1989; Holoshitz *et al*, 1989; Kaufmann *et al*, 1990). T cell clones isolated from the synovial fluid of patients with inflammatory arthritis proliferated in response to Mb hsp65 to an extent greater than the patient's peripheral blood (Gaston, 1990). Similarly, mononuclear cells from synovial fluid of patients with juvenile chronic arthritis demonstrated a substantial proliferative response to purified fractions of hsp60 (DeGraeff-Meeder *et al*, 1991).

At a humoral level, patients with rheumatoid arthritis have been reported to possess IgG and IgA antibodies to Mb hsp65 at a level higher than found in samples from healthy individuals (Rudolphi *et al*, 1997; Tsoulfa *et al*, 1989). These responses have been mirrored in animal models of rheumatoid arthritis, for example, adjuvant arthritis in Lewis rats (DeGraeff-Meeder *et al*, 1990).

Current reports conflict as to whether these immune components are reacting to mycobacterial specific epitopes or to conserved hsp65 epitopes presented on the hsp65 proteins from different species. The T cell clones isolated by Gaston *et al* (1990) recognised epitopes at the NH<sub>2</sub> terminus of the protein that were not conserved between species. However, equally strong evidence persists to demonstrate that immune system components recognise conserved epitopes of the hsp65 (Handley *et al*, 1996; Krenn *et al*, 1996; Munk *et al*, 1989). It is most



likely that antibodies are raised against both species-unique and common cross-reactive epitopes (Ivanyi *et al*, 1983). Hsp60 cross-reactive antibodies are the more probable of the two classes to be involved in the pathogenesis of arthritis, possessing the potential ability to recognise autologous human hsp60 and to induce an immune reaction against human tissue. The existence of cross-reactive immune cells has been demonstrated by Boog *et al* (1992). A monoclonal antibody was developed that recognised an hsp65 epitope expressed by human and bacterial hsp65. This monoclonal antibody was able to bind to the synovial membrane of JCA patients. A monoclonal antibody with unique specificity for human hsp60 was also able to bind to the synovial membrane of these patients. This illustrated that levels of endogenous hsp60 are raised in the chronic inflammatory environment, and that these self antigens may then become targets for an immune response.

The substantial evidence linking the Mb hsp65 to arthritis has recently been opened to speculation by the presentation of major evidence that that questions the accountability of previous work in this field. Handley *et al*, (1996) showed that levels of antibodies to groEL (i.e. anti *E.coli* hsp65) were higher than antibody levels against Mb hsp65 in patients with rheumatoid arthritis. Jarjour *et al* (1991) argue against a role for autoantibodies to hsp65 in rheumatoid arthritis, by claiming that these antibodies could not be detected in patients with rheumatoid arthritis. Furthermore, they found titers to bacterial hsp65 were so low that their role in the pathogenesis of rheumatoid arthritis could be disputed. It has been suggested that previous interpretations of ELISA for antibodies to Mb hsp65 in rheumatoid arthritis patients should be completely re-evaluated,

after the finding that IgM rheumatoid factor (present in patient sera) could lead to false-positive ELISA results by cross-linking of the primary and secondary antibodies used in the technique (Lim and Sharif, 1996). Removal of IgM RF from sera significantly reduced ELISA OD levels. Recently it has also been shown that hsp60 levels alter with age (Faulds *et al*, 1995). As this has not been accounted for in the majority of rheumatoid arthritis antibody studies, this again queries the viability of previous work.

### ***Hsp60 and immunodominance***

It has been suggested that the strength and uniformity of immune responses to hsps is due to the immune system being pre-programmed to react to this, and other, immunodominant antigens (Cohen, 1992; Cohen and Young, 1991). This would explain why healthy control patients have hsp reactive B and T cells. Having an immune response to dominant antigens that is pre-programmed has various advantages: it reduces the chaos element of developing an immune repertoire, and dominance to selected self antigens creates essential self tolerance to other self antigens due to their being part of a field of weak 'background' antigens. For these programmed immune cells recognising dominant antigens there is also a network of ready-made connections to the rest of the immune system, allowing both control and homeostatic regulation of responses, and also allows for ready-made T-help. This instantly available T-help means that programmed B and T cells recognising microbial hsp elicit a stronger, faster immune response than do other competing exogenous antigens. An efficacious immune response to hsp confers obvious advantages, considering the ability of hsps to be used as a common antigenic flag expressed

by invading micro-organisms and by inflamed and damaged host tissue.

Cohen's attractive theories go a long way to explaining many of the features pertinent to hsp 60s ie. their potent immuno-stimulatory powers, immunodominance, and widespread expression of hsp60 reactive T and B cells in vast range of disease patient groups and healthy controls. His ideas also greatly assist the onerous task of interpreting much of the conflicting and ambiguous papers presenting hsp studies.

### ***Hsp60 and autoimmunity***

As previously mentioned, hsp60 is expressed in virtually all cell types and is essential for cell viability (Zeilstra-Ryalls *et al*, 1991). As such, it is expressed constitutively in bacterial cells and in the mitochondria of human cells. Despite its apparent intracellular localisation, mammalian hsp60 has been found to be expressed on cell surfaces (Jarjour *et al*, 1990; Soltys and Gupta, 1996).

Antibodies against conserved epitopes of hsp65 have been shown in innumerable studies to be highly cross-reactive (Handley *et al*, 1996; Jones *et al*, 1993; Nakano *et al*, 1995). Hsp60 presents as an ideal candidate for stimulating an autoimmune response via antigenic mimicry: an invading organism presents elevated levels of extremely immunogenic hsp 60 antigen, against which an immune response is quickly launched. The elevated temperature of the site of infection, or any concomitant inflammatory reactions stress self cells, resulting in elevated levels of endogenous hsp60 also being expressed at the immune reactive site. Those T cell clones and antibodies raised against conserved sites of the stimulating hsp60/65 can potentially bind

the shared epitopes on the self antigen, and an auto immune reaction is initiated. Theories similar to this are currently cited in literature linking hsp60 to autoimmune disease. According to this concept, the overwhelming presentation of shared hsp epitopes to the immune system would eventually break down self tolerance to self hsp, and auto immune disease would ensue.

The existence of reactive T cells and antibodies that recognise self hsp has been demonstrated by many groups studying a range of diseases (Ellis and Vandervies, 1991; Handley *et al*, 1995; Kaufmann *et al*, 1990; Kaufmann, 1994; Kiessling *et al*, 1991). These studies, mostly immunoassays, have been further bolstered by the development of new techniques that allow elution and subsequent identification by microsequencing of peptides naturally processed and presented by major histocompatibility complex (MHC) gene products (Jardetsky *et al*, 1991). These studies have proved conclusively that hsp derived peptides are presented through both MHC class I and II pathways, and that it is often a conserved region of the hsp that is presented. Along with the identification of T cell clones that recognise synthetic peptides representing 'shared' hsp domains (Kaufmann *et al*, 1990), these data indicate that self hsp epitopes can be targeted by an immune response. This goes well to support the idea that such an immune response would cause auto immune disease – were it not that healthy control patients appear to possess auto-immune T cell clones and antibodies with the same specificity for self hsp (Handley *et al*, 1996; Munk *et al*, 1988; Munk *et al*, 1989).

Autoimmunity cannot be assumed to be indicative of auto immune disease. In a

view put forward by Cohen and Young (1991), autoimmune reactions are necessary to the host for the recognition and removal of aberrant host cells. As hsp expression is known to increase after a variety of cellular insults, for example during inflammation, transformation or infection, it appears as an ideal universal marker of stressed cells. Hsp reactive T cells have also been shown to recognise activated immune cells (Koga *et al*, 1989), and Cohen suggests that this reaction may be part of a homeostatic pathway to down-regulate immune responses, preventing immune hyper-reactivity. The non-pathogenic nature of an immune response to hsps is further supported by evidence indicating that some  $\gamma\delta$  T cell populations have a particular predilection for hsps. A high proportion of  $\gamma\delta$  T cells develop extra-thymically, and thus avoid thymic silencing of lymphocytes that are self reactive (Haas *et al*, 1993).  $\gamma\delta$  T cells are also thought to have a limited diversity of antigen recognition genes (Asarnow *et al*, 1988). As studied in the mouse, many  $\gamma\delta$  T cells are found localised in the epithelial layer. As such, they represent the first barrier an invading pathogen has to overcome when attacking the host (Asarnow *et al*, 1988; Janeway *et al*, 1988; Janis *et al*, 1989). Their role is principally thought to be one of immune surveillance, and it follows that they should have the ability to recognise any stressed, aberrant or infecting cell. As mentioned previously, hsp expression is a perfect 'flag' for stressed cells, whether microbial or of host origin, and  $\gamma\delta$  T cells have been widely demonstrated to recognise this ubiquitous antigen (Fisch *et al*, 1990; Haregewoin *et al*, 1989; Kaufmann and Kabelitz, 1991; Saito *et al*, 1997).

Collectively, these reports highlight the fact that autoimmune reactions to self

hsp are not necessarily detrimental to the host; rather, they are probably of significant benefit to the host. This has caused a recent re-evaluation of the link between hsps and autoimmune disease. It is still commonly held that there is a connection, as can be confirmed by the finding of sequence homologies between hsp60 and other known auto antigens (Jones *et al*, 1993). However, the identification of hsp60 reactive lymphocytes within autoimmune lesions is as likely to be representative of a healthy homeostatic down-regulation of acitivated immune cells or a primary response to infection as it is an indicator of self destructive processes.

Hsp reactive lymphocytes are more likely to feature in autoimmune disease when the healthy anti-hsp immune response becomes imbalanced, or homeostatic control of it fails (Cohen, 1992; Cohen and Young, 1991). Antigenic mimicry of hsp60s could be responsible for initiating autoimmune disease, if an aggressive immune reaction to exogenous bacterial hsp60 antigens was not properly controlled by suppressor elements of the immune system, and the aggressive reaction was misdirected to host organ-specific antigen. This would account for the organ-limited, as opposed to systemic, nature of autoimmune diseases.

Alternatively, hsp60 cross-reactive lymphocytes may arrive at the afflicted site secondarily, after inflammation had already been established (Anderton *et al*, 1993). In this situation stressed host cells of the target organ could become targets for an anti-hsp60 immune response. Again, if homeostatic control of the immune response to hsp60 failed, antigenic mimicry could lead to autoimmune

disease. As more organ-specific tissue was challenged by the immune response, host cells would face further stress and increased production of self-hsp60, perpetuating the cycle of self-attack.

### ***Hsp60 as diagnostic and prognostic indicators***

As previously discussed, the role of hsp60 in the pathogenesis of disease has recently been reviewed and the current ideas on the concept are still speculative. Critical reviews by Jarjour *et al* (1991), Tishler and Shoenfeld (1996) and Lim and Sharif (1996), have questioned the viability of previous studies which were quick to brand hsp60 as a pathogenic protein after an immune response to hsp60 had been demonstrated. Perhaps because of this, recent papers that have highlighted antibody responses to hsp60 in patient groups have been cautious with the interpretation of their findings. Rather than attempt to implicate these hsp60 specific antibodies as being involved in a pathogenic role in autoimmune disease, Gruber *et al* (1996) have instead used hsp60 antibodies to identify and define patient sub-groups within a large disease group. Studying patients with vasculitis, they suggest that the identification of a sub-group of patients that has raised antibody titres to mycobacterial hsp65 may highlight vasculitis cases that have an underlying bacterial infection of the arteries. They suggest that the presence of antibodies to Mb-hsp65 could distinguish those patients within the vasculitis group that have (or have had) an underlying infection of the arteries.

Portig *et al* (1997) proposed the same in their study on patients with cardiomyopathy, and also suggest that after the patient sub-group had been

defined, (i.e. those with high titers to hsp60), therapy could be targeted more effectively with specific anti-inflammatory treatment.

Investigation of antibodies to hsp60 in patients with rheumatoid arthritis persists. Prakken *et al* (1996), suggest that hsp60 reactive antibodies be used for the prognosis of oligoarticular juvenile rheumatoid arthritis, after they found that patients with a positive antibody response to human hsp60 developed disease remission within 12 weeks. Similarly, Peeling *et al* (1997) found that the presence of antibodies to recombinant chlamydial hsp60 within a group of 280 female sex workers in Africa predicted a 2-3 fold increased risk for chlamydial pelvic inflammatory disease, and it is suggested that the chlamydial hsp 60 antibodies can be an aid to prognosis of this disease.

Initial work has been carried out to identify antibodies to hsp60 in periodontitis and gingivitis patients that may aid diagnosis (Schett *et al*, 1997). Both these groups, and healthy patients, carried anti-hsp antibodies in their sera, but only the gingivitis group had a high level of antibodies against a cross-reactive hsp60 epitope in their saliva. Schett and colleagues propose the measurement of levels of salivary anti-hsp60 as a diagnostic marker for gingivitis, whilst high serum antibody titres to hsp60 are more frequently associated with periodontitis.

### ***Hsp60 and vaccines***

Hsp60s have been the focus of interest in vaccine development for two reasons: i) the development of anti-hsp vaccines to treat disease in which hsp 60 immune reactivity is thought to play a role (Prakken *et al*, 1998) and ii) use of hsps as stimulatory adjuvants for the development of novel vaccines in other



unrelated diseases (Jindal, 1996).

It has been demonstrated that bacterial hsps have the ability to prevent experimental arthritis in animal models following prior immunisation (Bloemendal *et al*, 1997). The protective mechanism of these vaccines is thought to be via induction of tolerance or via the induction of regulatory T cells that function in re-establishing homeostatic control of immune reactions against hsp60. Oral administration of a potential vaccine was shown to have a far better protective capacity than parenteral delivery of the same vaccine. This is thought to be due to the vaccine stimulating proliferative responses in different T cell types. Stimulation of T-helper 1 (Th1) T cells provokes an aggressive, non-protective response, whereas Th2 T cells, stimulated by oral administration of the vaccine, are regulatory (Tian and Kaufman, 1998).

By increasing numbers of regulatory cells (eg. anti-idiotypic cells) the autoimmune effector cells could be 'reigned-in' and healthy control of the immune response against hsp60 re-established (Cohen, 1992). Presumably altered T cell lines capable of aiding immune control could be produced *in vitro* and supplied as a passive vaccine, eliminating the risk of producing an aggressive response that may result from vaccination with bacterial hsp60 antigens directly.

The immunogenicity of hsp 60s and their ability to elicit a rapid immune response has caused a recent investigation into the ability of hsps to act as adjuvants (Jindal 1996; Lussow *et al*, 1991). Initial results show that hsp60 of *E. coli* and *M. bovis* behave as carrier molecules that can induce anti-peptide antibodies (in mice) without requiring the presence of other adjuvants.

Further work is necessary to assess the risk of stimulating responses against self-hsp with these vaccines, but it seems that there is potential for the development of vaccines against novel peptides by fusing peptides to hsp 60s via recombinant technology. This is creating interest in tumour research, with peptides associated with tumour cells being used to develop vaccines against homologous tumours.

### ***Autoimmune processes in periodontal disease***

As mentioned previously, human periodontitis is an infectious process mediated by a wide range of Gram negative bacteria (Moore and Moore, 1994). The infection leads to chronic inflammation which results in degradation of the supporting structures of the teeth, and eventually tooth loss.

Periodontal disease describes a manifestation of symptoms that are similar between patients. Numerous models for the pathogenesis of the disease have been proposed, but no definite evidence currently exists to support any one particular hypothesis as the periodontal paradigm. It seems likely that for a disease as complicated as periodontal disease is, and involving as many potential pathogens and risk factors as it does, that there is no one true cause or course for the disease and that many pathogenic processes occur concomitantly.

A role for autoimmunity in periodontal destruction was implicated over 25 years ago. It was first realised that cellular immunity played a role in periodontal destruction in 1970, when lymphocytes were shown to respond to bacterial antigens present in periodontal disease (Ivanyi and Lehner, 1971). Also,

periodontal disease has been seen to share characteristics with other inflammatory diseases in which autoimmunity is thought to be a component of the disease progression (Snyderman and McCarty, 1988). Whilst the idea of autoimmunity as a factor in periodontitis is still not conclusive, studies continue to support this concept and more sophisticated autoimmune models are proposed as we better understand the function and capabilities of immune cells and the effector molecules they express.

Anusaksathien and Dolby (1991) give a review of reports yielding evidence of autoimmunity in periodontal disease. A pertinent feature of many of these studies is the detection of 'autoantibodies', that is, antibodies that have been found to be reactive with self antigens. For example, autoimmunity to collagen has long been held as a pathogenic reaction that may be fundamental to periodontal disease. In a study by Hirsch *et al* (1988) autoantibodies to denatured collagen were detected and implicated in periodontal disease. As was previously mentioned though, the detection of autoantibodies is not sufficient to proclaim that autoimmune disease is occurring. It is possible that the autoantibodies detected play a benign, if not beneficial, role to the host in the disease process.

For some considerable time the idea of 'molecular mimicry' has been popularly cited as a likely cause of autoimmune reactions. Briefly, this theory holds that a bacterial antigen acts as the autoimmune trigger via cross-reactivity with self antigens. Antibodies raised against the exogenous protein are thus able to bind to the cross-reactive endogenous protein, and self-attack is initiated.

Various antigens have been mooted as bacterial molecular mimics of self proteins, including the hsp60s of bacteria. Hsp60s fulfil many criteria that deem them fit as archetypal molecular mimics, although they are less mimics than they are true homologues of a human protein.

### ***Heat shock response of periodontopathogenic bacteria***

The production of heat shock proteins is a crucial response to stress that protects cells exposed to any of a variety of insults, including exposure to elevated temperatures. The proteins allow cells to survive in unfavourable conditions, but have also been shown to be elemental for cell survival in normal conditions (Craig *et al*, 1993) and aid survival of bacterial pathogens when invading a mammalian host during infection (Buchmeier and Heffron, 1990). Several periodontopathogenic bacteria have been shown to respond to stress by eliciting a heat shock response. Generally cells were incubated at 37°C, and then subjected to 'heat shock' at 42°C for up to 20 minutes. SDS-PAGE analysis of the heat induced proteins revealed increased synthesis of a range of proteins from which hsp 60 homologues can be identified by Western immunoblotting.

The heat shock response has been illustrated for a number of known periodontopathogenic bacteria. These include strains of *Porphyromonas gingivalis* (Lu and McBride, 1994), *Actinobacillus actinomycetemcomitans* (Koga *et al*, 1993), *Treponema denticola* (Stamm *et al*, 1991), *Bacteroides forsythus*, *Prevotella oralis*, *Fusobacterium nucleatum*, *Porphyromonas endodontalis*, *Prevotella nigrescens*, *Prevotella melaninogenica*, *Treponema socranskii* and

*Prevotella intermedia* (Ando *et al*, 1995; Vayssier *et al*, 1994).

### ***Environmental conditions in the periodontal pocket***

Thermal shock, pH decrease, increase in oxygen concentration and mechanical stress have all been shown to induce the synthesis of hsp60 in oral bacteria (Vayssier *et al*, 1994; Yoshiyuki *et al*, 1999). The environmental conditions in the infected periodontal pocket are such that bacteria could be exposed to these stresses. The subgingival temperature is increased at diseased sites as compared to healthy ones (Fedi and Killoy, 1992; Kung *et al*, 1990), the pH of diseased sites decreases as periodontal pocket depth increases (Bickel and Cimasoni, 1985) and bacterial hsp60 levels increase after exposure to oxygen metabolites of phagocytes (Buchmeier and Heffron, 1990).

### ***Cross-reactive hsp60 antibodies present in periodontitis***

The hsp60 of several periodontopathic Gram negative bacteria have been identified as cross-reactive antigens. This has been suggested by sequencing the hsp60 genes and aligning the data with other hsp60 sequences (Maeda *et al*, 1994; Nakano *et al*, 1995; Reid and Riggio, 1998) to display the high level of sequence conservation shared amongst the proteins of different species, and verified by demonstrating that heat induced 60kDa proteins of periodontopathic bacteria can be detected by cross-reactive hsp60 antibodies (Ando *et al*, 1995; Hinode *et al*, 1998; Vayssier *et al*, 1994).

Heat shock proteins and the stress response of numerous prokaryotic and eukaryotic organisms have been widely studied as previously mentioned.

However, only a few research groups have focused on the heat shock proteins and stress responses of oral bacteria associated with periodontal disease. To date, the DNA sequences of the hsp60 genes from three periodontopathic bacteria have been published: *Actinobacillus actinomycetemcomitans* (Nakano *et al*, 1995), *Porphyromonas gingivalis* (Maeda *et al*, 1994), and *Bacteroides forsythus* (Reid and Riggio, 1998). The sequences share up to 85% sequence conservation throughout the entirety of the gene. Aligning these sequences with the hsp60 DNA sequences of other species shows that the oral pathogens have a level of homology of about 50-80% with other prokaryotic micro-organisms, and approximately 48% homology with the human hsp60 homologue, mitochondrial protein P1. Particular regions of the hsp60 DNA sequence appear to be extremely well conserved, and it could be that antibodies raised against these omnipresent epitopes would be highly cross-reactive, theoretically possessing the ability to react with virtually any other hsp60.

That hsp60 antibodies can cross-react with oral pathogenic micro-organisms has been established in several immunoblot analyses (Ando *et al*, 1995; Hinode *et al*, 1996; Hinode *et al*, 1998; Koga *et al*, 1993; Maeda *et al*, 1994; Nakano *et al*, 1995; Reid and Riggio, 1998). Typically, Western blots are probed with monoclonal antibodies raised against hsp60 of a non-periodontopathic organism (e.g. against hsp60 of *Yersinia enterocolitica* in Ando *et al*, 1995; *Mycobacterium leprae* in Nakano *et al*, 1995; *E. coli* in Reid and Riggio, 1998).

More significant are the Western blots that use monoclonal antibodies raised against periodontopathic bacterial hsp60 as a probe to detect hsp60 in other

periodontopathic bacteria though, as the high antibody titres at periodontally diseased sites will be composed of antibodies against the periodontopathogens, and not antibodies against a species that is not implicated in the disease. Thus, if an immunoblot can evidence that antibodies raised against an oral pathogen can cross-react with other oral pathogens, presumably this situation could be mirrored *in vivo* and could be of importance in the pathology of the disease.

Interestingly, monoclonal antibodies against fragments of the hsp60 protein of *A. actinomycetemcomitans* were shown to cross-react with several bacteria, but there was no reaction evidenced against the known periodontopathogens *Porphyromonas gingivalis*, *Prevotella intermedia* or *Fusobacterium nucleatum* (Nakano *et al*, 1995). Hinode *et al* (1998) raised polyclonal antibodies to the hsp60 proteins of *A. actinomycetemcomitans* and *P. gingivalis*. These antibodies cross-reacted with other hsp60 proteins, including those of *B. forsythus*, but notably not with human hsp60 protein. In direct contradiction to Nakano's study, Hinode's group showed that *A. actinomycetemcomitans* hsp60 antibodies did cross-react with *P. gingivalis* hsp60. This discrepancy in results may be due to Nakano using monoclonal antibodies to portions of the *A. actinomycetemcomitans* hsp60 as his immunoblot probe. Whilst monoclonal antibodies have an indisputable importance in research, in this study they were limiting in that they may have been raised to non-conserved epitopes, and would not be a true reflection of what may be occurring in the natural polyclonal responses of a patient.

### ***Presence of hsp60 reactive antibodies in sera from periodontal disease groups and healthy controls***

Having established that antigens raised against hsp60 are cross-reactive and have sufficient avidity to recognise hsp60 from other unrelated species, this work would take on further import should it be demonstrated that the hsp60 cross-reactive antibodies can be detected at higher levels in serum samples from periodontitis patients as compared to healthy controls.

Koga *et al* (1993) obtained serum samples from 21 patients with localized juvenile periodontitis or rapidly progressive periodontitis. Of these, 9 samples reacted with the 64kDa protein of *A. actinomycetemcomitans* (serotype b), as analysed by Western blotting. In the same study, sera taken from 10 periodontally healthy subjects showed no reaction.

Ando *et al* (1995) demonstrated the presence of antibodies that reacted with bacterial hsp60s of *A. actinomycetemcomitans*, *Fusobacterium nucleatum* and *Prevotella nigrescens* in one serum sample from a periodontitis patient. Further work was done to detect the immune response to hsp60 occurring in gingival tissue sections from 4 patients with adult periodontitis. Of the 4 tissue sections, 3 reacted with monoclonal antibodies against human hsp60 but did not react with bacterial hsp60. The same results arose when homogenised gingival samples were utilised. The group suggests that the discrepancy between the results from serum and tissue samples could be due to the periodontopathic bacterial hsp60 antigens forming large immunocomplexes with their relevant antibodies *in situ*. The formation of antigen-antibody aggregates would preclude the binding of any further hsp60 antibodies that would be necessary for a positive result in an immunoassay. These immunocomplexes would activate the



complement system and cause inflammation of the gingival tissue which, in turn, would cause an increased production of human hsp60 in host cells (as was detected in the gingival tissue).

A further study of hsp60 antibodies present in human periodontitis patient serum was performed when recombinant *P. gingivalis* hsp60 was purified and contested with sera from ten periodontitis patients with antibody titres elevated to *P. gingivalis*. Eight of the test group sera reacted to the *P. gingivalis* hsp60. Three out of nine healthy patient samples showed a positive reaction as well (Maeda *et al*, 1994).

This study possesses the advantage over the other serum studies referred to of utilising purified recombinant protein. The gene expressing *P. gingivalis* hsp60 had been cloned into an expression vector prior to purification of recombinant protein. This allowed the gene to be sequenced and conclusively proven to be the gene encoding hsp60. This level of certainty cannot be achieved by other groups that identify hsp60s, prior to purification, with immunoblot assays using antibodies to other hsp60s as a detective probe. Hsp60 antibodies are frequently shown in the relevant literature to possess the ability to bind to a number of proteins of varying size. These proteins may be hsp60 breakdown products, but the possibility remains that they could be unrelated proteins hybridizing to the hsp60 probes. Should this be the case, proteins could be purified and incorrectly identified as hsp60.

More recently, Tabeta *et al* (1999) synthesised recombinant *P. gingivalis* hsp60 to analyse the presence of reactive antibodies in serum and gingival tissue extracts from ten periodontitis patients and ten healthy controls. Most of the

diseased serum samples carried hsp60 antibodies, as did half of the tissue samples.

### ***Overview of hsp 60 in periodontal disease***

In conclusion, a number of separate studies have shown that:

1. The area of inflammation at a periodontally diseased site provides an environment that induces increased synthesis of human hsp60.
2. Periodontopathogenic bacteria typically produce increased amounts of hsp 60 following cellular stress.
3. Antibodies to bacterial periodontal pathogens and human hsp60 are cross-reactive.
4. Antibodies to bacterial and human hsp60 have variably been demonstrated to be present in gingival tissue sections and serum samples from patients with periodontal disease.

Antibodies to hsp60 have elicited cross-reactive responses in multitudinous studies, and it is not surprising that the same case was upheld for antibodies raised against hsp60 from periodontopathogenic bacteria. Progressions from these studies should lead to the identification of precise epitopes that yield either specific or cross-reactive antibodies.

More directly implicating a role for hsp60 and the antibodies it can potentially raise in periodontitis are the studies that substantiate evidence for the presence of hsp60 antibodies in periodontal diseased tissue and serum taken from afflicted patients. Only small numbers of patient/healthy subject samples have

been studied so far, with results based on groups of 1-21 patients. Hsp60 antibodies have been detected in healthy controls as well as in periodontitis patient serum, although at a lower prevalence (Ando *et al*, 1995; Maeda *et al*, 1994; Schett *et al*, 1997; Tabeta *et al*, 1999). The significance of these results is difficult to determine though as statistical interpretation of the data is problematic due to the small sample numbers used. Also, no two studies use identical antibodies for their studies. Replication of results has not yet been demonstrated between groups, and the differing studies cannot be analysed *en masse* due to differences in study design and materials used. It is essential that these factors be addressed before a consensus on the work done by the various groups can be considered.

From the available data, it appears that cross-reactive antibodies to hsp60 can be found in healthy as well as periodontally diseased subjects ( Maeda *et al*, 1994; Nakano *et al*, 1995; Tabeta *et al*, 1999) although some reports do not detect hsp60 reactive antibodies in healthy samples (Ando *et al*, 1995; Koga *et al*, 1993). The conflict in these studies may be resolved if larger numbers of subjects are studied. Another factor contributing to the apparent discrepancy may be patient age (Faulds *et al*, 1995). Levels of antibodies to hsps have been shown (in mice) to increase with age, however, none of the mentioned reports note the age of the patients from which serum samples or gingival tissue have been extracted.

As discussed previously, in the case of many other diseases in which hsp60 is thought to be involved in the pathogenesis, hsp60 antibodies have been found

in healthy controls also (Handley *et al*, 1995; Munk *et al*, 1988; Munk *et al*, 1989). Thus one could presume that the same situation will be described when more data is available to compare healthy subjects with periodontitis samples. This finding would not completely rule out hsp60 antibodies as factors involved in periodontal disease pathogenesis however. An explanation for the seemingly ubiquitous human response to hsp60 of human and bacterial origin has been postulated by Cohen (1992). Briefly, he suggests that a pre-programmed immune response to hsp60 is instilled within us all. Hsp60, he suggests, is a universal marker that permits a) recognition of damaged, infected or stressed human cells and their subsequent removal, and b) a rapid ready-prepared response to invading organisms. To modulate and keep homeostatic control of the immune response to hsps it is necessary that there are control elements of the immune system dedicated to down regulating the immune response to hsp 60. When there is a failure of this regulation there may be a pathogenic autoimmune response to self-hsp60 molecules. The pathogenic autoimmune response may lead to initiation or perpetuation of autoimmune disease. To cause disease, the effector mechanisms active against exogenous hsp60 which facilitate the obliteration of invading micro-organisms could, if not sufficiently controlled, turn their potent actions against an organ specific self antigen. This self-antigen could be self hsp60 expressed at high levels on stressed localised tissue. An intriguing finding is that periodontal ligament fibroblasts have DNA sequences homologous to hsp60 (Gemmell and Seymour, 1995). As an antigen, this is specific to the periodontium and is perfectly localised to fulfil an explanation of the organ specific nature of periodontal disease. Should these cells be the target of an autoimmune attack, increasing numbers of host cells

would be exposed to stress that would induce a further increase in hsp60 synthesis, and mark further numbers of host cells for recognition by hsp60 specific T-lymphocytes and antibodies. Chronic stimulation of this calamitous cycle would inevitably result in the destruction of tissue and bone which is characteristic of periodontitis.

A review of the cellular immune response in periodontitis lends some support to this idea. Information on cells removed from periodontal lesions suggests that there is an upset in local immunoregulation (Seymour *et al*, 1996). Also, early indications are that Th2 cells may be dominant in periodontitis lesions (Genco and Slots, 1984; Seymour *et al*, 1996). A crucial function of Th2 cells is to provide help to B cells secreting antibodies, which would implicate chronic antibody production in the pathogenesis of this affliction.

Bacterial hsp60 homologues may have a role other than one as immunogens in the pathogenesis of periodontitis. A report has presented data, based on sequence analysis and electron microscopic analysis of protein preparations, that an hsp60 homologue isolated from *A. actinomycetemcomitans* interacts directly with bone cells and induces bone resorption (Kirby *et al*, 1995). The hsp60 homologue was present in the surface associated material of the bacterium, and was isolated by a saline extraction procedure. Sequencing of the NH<sub>2</sub> terminal of the protein revealed 36 out of 38 residues to be identical to the *E. coli* hsp60, and approximately 60% conservation shared with the human hsp60 homologue, mitochondrial protein P1. A preparation of the protein was also examined by electron microscopy, and a double ring structure

characteristic of hsp60 was observed. The protein possessed extremely efficacious osteolytic activity, demonstrating resorbing activity at ~1pM molar concentration.

In conclusion, current literature suggests that bacterial hsp60s and antibodies to these proteins may be involved in the pathogenic processes of a wide range of infectious diseases and autoimmune conditions. The involvement of hsp60 in periodontal disease is currently being investigated as a number of findings have shown that hsp60 antigens from periodontopathogenic bacteria can raise a cross-reactive immune response in the host which may be significant in disease progression. This study aimed to further work in this field by studying the hsp60 of the periodontal pathogen *B. forsythus*. It was aimed that this should be done by ascertaining the molecular identity of the gene encoding the protein, which would indicate the level of genetic homology shared between this species and others, and would also allow an analysis of the predicted amino acid sequence of the protein. Following this, it was planned that an initial characterisation of the immune response to this protein could be facilitated by production of recombinant *B. forsythus* hsp60 and by using this recombinant to screen serum samples from healthy individuals and from patients with periodontitis to measure antibody responses.

## **CHAPTER 2**

### **General Materials and Methods**

## **MATERIALS**

This chapter describes materials and methods used throughout the studies presented in this thesis. Methods that specifically apply to work covered in individual chapters are detailed in the relevant section of each chapter.

### **Materials**

#### **Bacterial strains**

*Escherichia coli* strains TOP10 and TOP10F': Invitrogen BV, 9704 CH Groningen, The Netherlands.

*Bacteroides forsythus* strain ATCC 43037.

*Prevotella intermedia* strain ATCC 25611.

#### **Plasmids**

Plasmid pUC18: Amersham Pharmacia Biotech, St. Albans, UK.

Plasmid pCR2.1<sup>®</sup>-TOPO: Invitrogen BV, 9704 CH Groningen, The Netherlands.

Plasmid pBAD<sup>®</sup>-TOPO: Invitrogen BV, 9704 CH Groningen, The Netherlands.

#### **Enzymes**

Restriction endonucleases: Promega, Southampton, UK and Gibco BRL, Life Technologies Ltd., Paisley, UK.

*Taq* polymerase: Promega, Southampton, UK.

T4 DNA ligase: Gibco BRL, Life Technologies Ltd., Paisley, UK.

Klenow: Amersham Pharmacia Biotech, St. Albans, UK.



## ***Chemicals***

Tris base, boric acid, ethidium bromide, sodium chloride, mineral oil, Triton X-100, spermidine, phenol, ampicillin, IPTG, X-gal, glycerol: Sigma-Aldrich, Poole, UK.

Ethanol, bromophenol blue, sodium acetate, magnesium chloride, sodium hydroxide, ethylene diamine tetraacetic acid (EDTA), sodium dodecyl sulphate (SDS): BDH Ltd, Poole, UK.

Formamide, agarose, tryptone, yeast extract: Gibco BRL, Life Technologies Ltd, Paisley, UK.

Tetramethylethylenediamine (TEMED), acrylamide, methylene bis-acrylamide, ammonium persulphate, Bind-Silane, Repel-Silane, urea, Rapid Gel XL: Amersham Pharmacia Biotech, St. Albans, UK.

Ready Red Chloroform Isoamylalcohol : Appligene Oncor Lifescreen, Watford, UK.

Arabinose: Invitrogen BV, 9704 CH Groningen, The Netherlands.

Lysozyme: Boehringer Mannheim

## ***Radioisotopes***

[ $\alpha$ - 35S] dATP: Amersham Pharmacia Biotech, St. Albans, UK.

### ***Other items***

Kodak X-ray film: HA West, Clydebank, UK.

Whatman 3MM paper: Whatman International Ltd, Maidstone, UK.

Nylon membrane, positively charged: Boehringer Mannheim, Lewes, UK.

### ***Kits***

QIAEX II Gel Extraction Kit: Qiagen, Crawley, UK.

Qiagen Plasmid Kit: Qiagen, Crawley, UK.

Qiafilter Plasmid Maxi Kit: Qiagen, Crawley, UK.

Wizard Plus Minipreps: Promega, Southampton, UK.

Puregene Bacteria and Yeast DNA Isolation Kit: Flowgen, Lichfield, UK.

Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP: Amersham Pharmacia Biotech, St. Albans, UK.

DIG DNA Labelling and Detection Kit: Boehringer Mannheim, Lewes, UK.

TOPO TA Cloning, Invitrogen BV, 9704 CH Groningen, The Netherlands.

### ***Equipment***

PerkinElmer DNA Thermal Cycler: Perkin Elmer Applied Biosystems, Warrington, UK.

EasyjecT Basic electroporator: Flowgen, Lichfield, UK.

Hybaid Midi Dual 14 oven: Hybaid, Teddington, UK.

LI-COR Gene ReadIR 4200 automated sequencer: MWG Biotech, Milton Keynes, UK.

ImageMaster VDS with Fujifilm thermal imaging system: Amersham Pharmacia Biotech, St. Albans, UK.

Template Tamer: Appligene Oncor Lifescreen, Watford, UK.

Hybaid Omnigene thermal cycler: Hybaid, Teddington, UK.

## **METHODS**

### ***Bacterial culture conditions***

*Bacteroides forsythus* ATCC strain 43037 and *Prevotella intermedia* ATCC strain 25611 were both incubated anaerobically (85% nitrogen, 10% hydrogen and 5% carbon dioxide) on Columbia Blood agar supplemented with 7.5% defibrinated horse blood. In addition to this, the *B. forsythus* medium contained N-acetylmuramic acid and the *P. intermedia* medium contained 0.5µg/ml vitamin K and 5µg/ml haemin. *B. forsythus* culture could typically be seen after 7 days incubation, and *P. intermedia* after 5 days.

### ***Isolation of bacterial DNA***

Bacterial DNA was isolated using the Puregene DNA Isolation Kit, following the procedure for Gram negative bacteria. All solutions were provided in the kit. Bacterial cells were harvested by scraping the surface of the agar plates upon which they had grown. The cells were then gently agitated in 600µl cell lysis solution until the cells were evenly suspended, and incubated at 80°C for 5 min to lyse the cells. The sample was then cooled to room temperature.

Contaminating RNA was removed by adding 3µl RNase A solution to the lysate and mixing the sample by inverting the tube 25 times. The sample containing the RNA-digesting enzyme was incubated at 37°C for 60 min and then cooled to room temperature. Contaminating cytoplasmic and nuclear proteins were then removed by salt precipitation. 200µl protein precipitation solution was added to the RNase treated sample and vortex mixed for 20 sec. The sample was then centrifuged at 14,000g for 3 min. Precipitated proteins formed a pellet, allowing the supernatant containing the DNA to be removed to a clean 1.5ml

tube. Genomic DNA was finally isolated by precipitation with alcohol. 600µl 100% isopropanol was added to the protein-free supernatant and the tube inverted 50 times to gently mix. Centrifugation at 14,000g for 1 min then caused the DNA to form a small white pellet in the bottom of the tube. The supernatant was removed, and the DNA pellet washed by inverting the tube upon addition of 600µl 70% ethanol. The tube was then centrifuged as previously. The ethanol was poured off and the DNA pellet left to air dry for 20 min. The dried pellet was re-hydrated overnight in 100µl dH<sub>2</sub>O, and the DNA was stored at 4°C.

### ***Production of plasmids***

All plasmids used carried an ampicillin resistance (Amp<sup>R</sup>) gene.

Transformed *E. coli* (strain varied depending on procedure) was inoculated into 5ml L-broth containing 100µg/ml (final concentration) ampicillin. The bacteria were then cultured by incubating overnight in an orbital shaker, 200 rev/min, at 37°C. Glycerol stocks of the bacteria were made by taking 0.5ml of culture and adding to it 0.5ml 50% glycerol. These stocks were maintained at -80°C until required.

For large scale isolation of plasmid DNA, 1ml of the overnight culture was inoculated into 500ml L-broth containing 100µg/ml ampicillin. This was incubated as previously. The following day, bacterial cells were harvested by centrifugation in a Sorvall SLA-1500 rotor at 10,000 rpm for 30 min at 4°C.

## ***Isolation of plasmid DNA***

### ***Large scale***

Plasmid DNA was isolated on a large scale to replenish parental plasmid stocks, and also was the method of choice to isolate plasmids carrying gene fragments prior to manual sequencing of the gene.

For large-scale preparations, the Qiagen Plasmid Purification kit was used, following the QIAfilter Maxi protocol. This protocol did not require centrifugation of bacterial lysates, using QIAfilter cartridges (supplied in the kit) instead. The procedure is therefore quicker than conventional ones. QIAGEN-tip 500s and all solutions were also supplied in the kit.

After the bacterial culture had been pelleted the pellet was resuspended in 10ml Buffer P1 containing RNase A. Once the cells were evenly resuspended, 10ml Buffer P2 was added and the sample mixed by inverting 5 times. The sample was then incubated at room temp for 5 min to allow lysis to occur. 10ml chilled Buffer P3 was added to the lysate, gently mixed by inverting, and immediately transferred into the barrel of a QIAfilter Cartridge (with outlet nozzle attached). Without inserting the cartridge plunger, the sample was left at room temp for 10 min. Meanwhile, a QIAGEN-tip 500 was equilibrated by application of 10ml Buffer QBT which was allowed to drip through the column by gravity flow. The plunger was inserted into the QIA filter cartridge and the filtered cell lysate dripped into the equilibrated QIAGEN-tip. The lysate was left to enter the resin by gravity flow.

The QIAGEN-tip was then washed by passing 2X30ml Buffer QC through the resin. Following the washes, DNA was eluted with 15ml Buffer QF and the eluate collected. DNA had then to be precipitated by addition of 0.7 volume

isopropanol. The sample was quickly mixed and centrifuged in a Sorvall SS-34 rotor for 30 min at 10,000g, 4°C. The supernatant was removed and the remaining pellet washed with 70% ethanol. The sample was centrifuged again for 10 min, supernatant removed, and the pellet air-dried for 15 min. Lastly, the DNA was resuspended in 200µl dH<sub>2</sub>O.

### ***Wizard Plus Mini-Prep***

Plasmid DNA to be sequenced was isolated from overnight cultures of bacteria using a Wizard<sup>®</sup> Plus Minipreps DNA purification system (Promega). Following the manufacturer's instructions, 3ml of bacterial culture was centrifuged for 2 min at 14,000 rpm. The resulting pellet was resuspended in 200µl cell resuspension solution (50mM Tris-HCl, pH 7.5; 10mM EDTA; 100µg/ml Rnase A) and the solution inverted 4 times to mix. The suspension of cells was then lysed with 200µl cell lysis solution (0.2M NaOH and 1%SDS) and the sample again inverted to mix. 200µl neutralisation solution (1.32M potassium acetate) was then added, and the mixture could be seen to have cleared instantly. Samples were then centrifuged at 14,000 rpm for 5 min. The cleared supernatant was added to 2ml Wizard<sup>®</sup> Minipreps DNA purification resin in a syringe column, and the slurry slowly pushed through an attached Luer-Lok<sup>®</sup> minicolumn. The minicolumn was then washed by passing 2ml column wash solution (80mM potassium acetate; 8.3mM Tris-HCl, pH 7.5; 40µM EDTA; 55% ethanol) through the column. The column was completely dried after washing by placing the detached minicolumn in a clean tube, and centrifuging at 14,000 rpm for 2 min. To elute DNA from the column, 30µl dH<sub>2</sub>O was applied to the column for 1 min prior to centrifuging in another clean tube for 20 sec.

dH<sub>2</sub>O to bring the mixture to the correct working concentration. Spermidine (1mM final concentration) was added to reactions containing Life Technologies-supplied enzymes, and bovine serum albumin (0.1mg/ml final concentration) added to reactions containing Promega-supplied enzymes.

Digestion reactions were thoroughly mixed and incubated at 37°C for no less than 3 hours. Digests could then be stored at -20°C until required.

### ***Agarose gel electrophoresis***

Agarose was dissolved in 1XTBE buffer (Table 1.1) to a final concentration of 0.8% or 2%, by heating in a microwave. Ethidium bromide (1µg/ml) was added to the molten gel. The agarose solution was poured into the electrophoresis gel box, the well-forming comb inserted and the gel left to solidify. When solid, the comb was removed and 1XTBE electrophoresis buffer poured into the gel box to cover the gel.

0.1 volume sample loading dye was added to each of the samples before they were loaded into the wells. Electrophoresis was typically performed by applying a voltage of 65V for 30 min-1 hour.

DNA was visualised on an ImageMaster®VDS (Amersham Pharmacia Biotech) and photographed with the Fujifilm thermal imaging system.

### ***DNA purification from agarose gels***

DNA was purified from agarose gels following electrophoresis to either a) isolate a discrete fragment of a specific size or b) to purify the DNA and remove contaminating enzymes and buffers. Two methods were routinely used:

i) Spin-X filter tubes



DNA was excised from the agarose gel using a sterile scalpel and the gel slices put into Spin-X filter centrifuge tubes. The tubes were frozen at -20°C for 20 min and then centrifuged at 14,000 rpm in a benchtop microcentrifuge. The eluate collected in the bottom of the tubes was transferred to a clean 1.5ml tube and the DNA extracted with an equal volume of phenol (1M Tris-HCl, pH 8.0 equilibrated)/chloroform (1:1), then 1 volume chloroform. Precipitation of DNA was performed by adding 2 volumes filter-sterilised 100% ethanol and 0.1 volume 3M sodium acetate, pH 6.0, and stored at -70°C for 30 min. The tubes were then centrifuged at 14,000rpm for 10 min, and the DNA pellet washed in 70% ethanol. The washed pellet was lyophilised and finally resuspended in 20-30µl dH<sub>2</sub>O. This was stored at -20°C until required.

#### ii) QIAEX II Gel Extraction Kit

The manufacturer's protocol for DNA extraction from agarose gels was followed. All components were contained within the kit.

As previously, DNA was excised from an agarose electrophoresis gel using a scalpel. The gel slice was weighed, and the appropriate amount of Buffer QX1 added to the tube. The amount of Buffer QZ1 to be added was also dependent on the size of the DNA fragment, and could be determined according to the following table:

DNA fragments <100bp	Add 6 volumes Buffer QX1 e.g: 600µl buffer per 100mg gel
DNA fragments 100bp-4kb	Add 3 volumes Buffer QX1
DNA fragments >4kb	Add 3 volumes Buffer QX1 plus 2 volumes H <sub>2</sub> O

QIAEX II was resuspended by vortexing for 30 sec. QIAEX II contains silica gel particles that adsorb nucleic acids, and was added to the sample as below:

≤ 2µg DNA	Add 10µl QIAEX II
2-10µg DNA	Add 30µl QIAEX II
Each additional 10µg DNA	Add 30µl QIAEX II

The agarose was solubilised by incubating the tube at 50°C. The sample was vortexed every 2 min to keep the QIAEX II particles in suspension. At the end of the incubation, the sample was centrifuged at 14,000rpm for 30 sec and the pellet washed once with 500µl Buffer QE. The washed pellet was air-dried and the DNA eluted with 20µl H<sub>2</sub>O as below:

DNA fragments ≤4kb	Incubate at room temp for 5 min
DNA fragments 4-10 kb	Incubate at 50°C for 5 min
DNA fragments >10 kb	Incubate at 50°C for 10 min

This was centrifuged for 30 sec, and the supernatant containing the DNA was transferred to a clean tube. The elution was repeated with another 20 $\mu$ l dH<sub>2</sub>O to increase the yield of DNA, and the pooled eluates stored at -20°C.

**Table 1.1. General Stock Solutions and Buffers**

***Luria Bertani (LB) broth***

Tryptone                    10g

Yeast extract            5g

NaCl                        10g

Make volume up to 1 litre with dH<sub>2</sub>O. Sterilise by autoclaving.

***10x TBE***

Tris base                108g

Boric acid               55g

0.5M EDTA            40ml

Make volume up to 1 litre with dH<sub>2</sub>O. Sterilise by autoclaving.

***Ethidium bromide (3mg/ml)***

Ethidium bromide    30mg

dH<sub>2</sub>O                        to 10ml

Store away from light and use at a final concentration of 1 µg/ml.

***Bromophenol blue loading dye***

Bromophenol blue    0.05% w/v

Glycerol                 50%

***1M Tris-HCl (pH 8.0)***

Tris base	121g
dH <sub>2</sub> O	900ml

Adjust to pH 8.0 with HCl. Make up to 1 litre with dH<sub>2</sub>O. Sterilise by autoclaving.

***STET buffer***

NaCl	0.12g
1M Tris-HCl	0.2ml
Triton-X 100	1.0ml
0.2M EDTA	10.0ml

Make up volume to 20ml with dH<sub>2</sub>O.

## **CHAPTER 3**

### **Identification and cloning of the *B. forsythus* hsp60 gene**

## INTRODUCTION

At the onset of the studies presented in this thesis the hsp60 gene of *B. forsythus* had not been cloned or sequenced. Data presented in this chapter confirms the presence of a hsp60 gene in *B. forsythus*, and details the isolation and cloning of this gene into a plasmid vector.

The hsp60 protein was chosen for study for several reasons. As discussed in Chapter 1 of this thesis, numerous studies of hsp60s have illustrated a role for hsp60 in bacterial immunogenicity, and a possible involvement in the pathogenicity of autoimmune diseases (Kaufmann, 1991, 1993, 1994; Kiessling *et al*, 1991; Shinnick, 1991; Winfield and Jarjour, 1991; Coates, 1996). It has been demonstrated that the protein can confer protective immunity (Bloemendal *et al*, 1997), and it has been mooted as a candidate for potential vaccine development (Young *et al*, 1987; Lussow *et al*, 1991; Jindal, 1996). There is a growing realisation that a comprehension of hsp60 antigens is not only important for our understanding of the immunopathology of bacterial infections, but may also expand our appreciation of immune surveillance and autoimmunity.

Of the periodontal pathogens, those most widely studied with regards to the hsp60 they express are *A. actinomycetemcomitans* and *P. gingivalis*. The gene expressing the hsp60 protein of *A. actinomycetemcomitans* was amplified by PCR, and this product cloned into a vector for subsequent DNA sequence analysis (Nakano *et al*, 1995). Chromosomal DNA of *P. gingivalis* was digested with restriction endonucleases and a fragment harbouring the hsp60 gene identified by Southern blot analysis using a labelled *E. coli* hsp60 gene probe. This fragment was then cloned into a plasmid vector prior to sequence analysis

(Maeda *et al*, 1994).

*B. forsythus* is a Gram negative, strictly anaerobic bacillus that has recently been strongly associated with periodontitis and may be a risk factor for particular forms of the disease. Studies on *B. forsythus* are more limited than those carried out on other periodontal pathogens, such as *P. gingivalis* and *A. actinomycetemcomitans*, which is probably due to the difficulties encountered in culturing this fastidious organism in the laboratory.

A 32-base pair fragment of the N-terminus of the hsp60 gene of *B. forsythus* has been characterised previously (Hinode *et al*, 1998), but there are no current reports of successful cloning or sequencing of the entire gene from this species. This chapter describes how the hsp60 gene of *B. forsythus* was identified, isolated and cloned into a plasmid vector. Cloning the gene circumvents the need to purify *B. forsythus* hsp60 by conventional methods that require large culture volumes (Hinode *et al*, 1996). The hsp60 gene was isolated by PCR, using degenerate oligonucleotide primers derived from the consensus of an alignment of hsp60 DNA sequences from other species. The identity of the resulting PCR product was confirmed by Southern blot analysis using a labelled *E. coli* hsp60 probe. Cloning of the gene was a crucial development that would allow further characterisation of the *B. forsythus* hsp60 gene and the protein it encodes.



## **MATERIALS AND METHODS**

### ***PCR amplification of hsp60 genes from various species***

#### ***i) Primer selection***

Primers were synthesised by Cruachem Ltd, Glasgow, and supplied as lyophilised pellets. Primers were resuspended in molecular biology grade water at a concentration of 1µg/µl. For amplification of the *B. forsythus* hsp60 gene, consensus primers were derived by alignment of known sequences of the groEL genes of several other species. The sequence of the consensus primer C1 is taken from Nakano *et al* (1995). The sequences of the consensus primers used were:

5'-ATTATGGCAAAAGAAATCAAATTCG-3' (PgN1)

and

5'-TTACATCATGCCGCCCA-3' (C1)

Primer PgN1 is a region of the *P. gingivalis* groEL gene that corresponds to nucleotides 45-70 of the *E. coli* hsp60 sequence. Primer C1 represents the 3' terminus of the groEL gene.

#### ***ii) Template DNA***

*B. forsythus* strain ATCC 43037 genomic DNA was used as the template for PCR. The primers were also used on genomic DNA extracted from *Prevotella intermedia*, *Prevotella nigrescens*, *Treponema denticola* and *Streptococcus mutans* to attempt the amplification of their groEL genes by PCR. Bacteria were cultured and genomic DNA purified as previously described.

### ***iii) PCR***

All solutions were handled in a designated PCR laboratory in a UV-sterilised microbiological safety cabinet, using filter pipette tips to prevent contamination of the samples. Template DNA was added using positive displacement pipettes in a sterile Template Tamer cabinet (Oncor Appligene, Watford, UK) as a further anti-contamination step, to prevent exposing the laboratory to aerosolised genomic DNA.

Lower and upper reaction master mixes were prepared separately on ice before aliquoting to prevent primer annealing and polymerisation occurring before the optimum designated temperature was attained in the thermal cycler. For the upper reaction mix, primers were at a final concentration of 0.2 $\mu$ M. The composition of the reaction mixes is shown in Table 3.1. The upper and lower reaction mixes were thoroughly mixed by vortexing and 50 $\mu$ l of the lower mix was aliquoted into each 0.5ml reaction tube. 50 $\mu$ l molten wax was pipetted on top of the lower mix and allowed to solidify, and then 49 $\mu$ l upper mix added to each tube. The wax separated the lower mix from the upper mix until a high temperature was reached in the thermal cycling reactions, and prevented any reactions from occurring before this temperature was attained. This 'hot start' PCR ensured that increased sensitivity and specificity of the reactions was obtained. Finally, 1 $\mu$ l (100ng) template DNA was added per tube. A negative control reaction was always used. This contained the same lower and upper mixes and wax as the reaction tubes, but contained 1 $\mu$ l molecular biology grade water in place of template DNA.

#### ***iv) Thermal cycling***

Thermal cycling was carried out in a Hybaid Omnigene thermal cycler (Hybaid, Teddington, UK). Cycling conditions were as follows:

(94°C, 1 min) 1 cycle

(94°C, 1 min; 55°C, 1 min; 72°C, 1.5 min) 30 cycles

(72°C, 10 min) 1 cycle

PCR products were either analysed immediately or stored at -20°C until required.

#### ***Southern Blot analysis of PCR products***

##### ***i) Southern transfer***

PCR products were electrophoresed on a 0.8% agarose gel as previously described, and visualised on a UV transilluminator.

Unused areas of the gel were cut away with a scalpel and the top left corner of the gel nicked to allow the gel to be easily orientated. The gel was placed in a glass dish and washed as described below. A description of the required solutions is given in Table 3.2.

1- 2x10 min washes with 250ml 0.25M HCl.

2- 2x15 min washes with 250ml Denaturation solution (to separate double stranded DNA).

3- 2 brief washes with 250ml dH<sub>2</sub>O to remove traces of Denaturation solution.

4- 2x15 min washes with 250ml Neutralisation solution (to prevent DNA reannealing).

All washes were performed at room temp with gentle shaking.

The gel was placed DNA side down on the permanent wick of the transfer unit.

The transfer unit consists of a shallow bench-top bath bridged by a porous material that is the wick. The bath holds 2xSSC solution which passes through the permanent wick, causing the transfer of DNA from the agarose gel to a nylon membrane. To ensure efficient DNA transfer, air bubbles trapped between the gel and the wick were removed by rolling a 10ml pipette gently over the gel surface.

Handling with sterile tweezers, a piece of nylon membrane was cut to the same size as the gel, soaked in 2xSSC and rolled on top of the gel and trapped air bubbles removed. Six pieces of 3MM Whatman paper were also cut to the same dimensions, soaked in 2xSSC and layered on top of the membrane, sandwiching the transfer membrane between the gel and the Whatman paper. A (3-inch) stack of folded paper towels was placed on top of the gel sandwich, the lid of the transfer unit put in place and a light weight applied to the top of the lid to ensure good contact between the gel and the membrane. 2xSSC was poured into the unit until level with the bottom edge of the permanent wick, and the unit left overnight. Following transfer, the unit was dismantled and the membrane carefully peeled off the gel using tweezers. The membrane (now the "blot") was then washed twice in 2xSSC to remove traces of agarose, blotted dry on Whatman paper and left to air dry for 10 min. The DNA was then immobilised on the membrane by crosslinking in a UV crosslinker (UVC-508; Anachem, Luton, UK). The blot was either used immediately for hybridisation to labelled DNA probe or kept in a sealed bag at 4°C until required.

## ***ii) DNA probe preparation***

An *E. coli* hsp60 probe was prepared using the Random Primed DNA Labelling

method, with Digoxigenin-11-dUTP as a label. Components for the labelling reaction were contained in the DIG DNA Labelling and Detection kit (Boehringer Mannheim, Lewes, UK) and are listed in Table 3.3. The *E. coli* hsp60 gene amplified by PCR was used as the DNA template in the labelling procedure. 1µg of DNA template was heat-denatured in a boiling water bath for 10 min and immediately chilled on ice. The template was then mixed with 2µl hexanucleotide mixture, 2µl dNTP labelling mixture, 1µl Klenow enzyme and sufficient molecular biology grade H<sub>2</sub>O to bring the volume to 20µl. The reaction was incubated at 37°C for 18 hours and then terminated by adding 2µl 0.2M EDTA. Labelled DNA was precipitated by adding 0.1 volume 4M LiCl and 3 volumes chilled 100% ethanol, mixing, freezing the reaction at -70°C for 30 min and centrifuging at 13,000 rpm for 15 min in a microcentrifuge. The pellet was isolated and washed with 100µl chilled 70% ethanol. After centrifugation at 13,000 rpm for 5 min, the supernatant was removed and the pellet lyophilised. The labelled DNA was resuspended in 50µl molecular biology grade H<sub>2</sub>O and stored at -20°C. From the starting amount of 1µg DNA template, 750ng of labelled DNA was normally generated.

### ***iii) PCR amplification of E. coli hsp60 gene probe***

The hsp60 gene of *E. coli* was generated by PCR to use as a probe in Southern blots. *E. coli* hsp60 primers N1 and C1 were used. These primers had been utilised previously by another group to successfully amplify the groEL genes of *E. coli* and *A. actinomycetemcomitans* (Nakano *et al*, 1995). The primer sequences were as follows:

5'-AATGGCAGCTAAAGACGT-3' (N1)

and

5'-TTACATCATGCCGCCCA-3' (C1)

Primer N1 corresponds to nucleotides 45-62 at the 5' end of the *E. coli* gene, and C1 corresponds to a region at the 3' terminus (nucleotides 1676-1692).

PCR was carried out using the same reaction conditions as described previously in this chapter. *E. coli* genomic DNA was used as the template for PCR. The bacteria were cultured and DNA isolated as described in Chapter 2.

#### ***iv) Hybridisation of labelled DNA probe to membranes***

The dried, cross-linked blot was sandwiched between 2 pieces of nylon mesh that had been cut to the size of the blot. This sandwich was submerged in 2xSSC, rolled up tightly and placed in a glass Hybaid rolling tube with 10ml 2xSSC. The bottle was rotated manually such that the blot unwound and covered the interior surface of the glass tube. The SSC was discarded and 20ml standard prehybridisation solution applied. The tube was placed in a Hybaid hybridisation oven (Hybaid, Teddington, UK) and rotated. Prehybridisation was allowed to proceed at 68°C for 3 hours.

The prehybridisation solution was discarded after this time and the labelled DNA probe applied. Immediately prior to use, 15µl labelled DNA probe (approximately 250ng DNA) was heat-denatured in a boiling water bath for 10 min and chilled on ice. The chilled probe was diluted in 10ml fresh standard hybridisation buffer (Table 3.2) before incubating with the membrane. The probe was left to hybridise to membrane-bound DNA at 68°C for 16-20 hours. After hybridisation, the probe was retained and stored at -20°C. The diluted probe could be re-used in 5 subsequent Southern blots. For re-use, the probe was

thawed and denatured by boiling as 10 min and used directly.

Unbound probe was removed from the membrane by washing the blot for 2x5 min in 2x wash solution at room temp. The blot was then washed for 2x15 min in 2xSSC/0.1%SDS with shaking at 68°C and processed directly for colorimetric detection of hybridised probe.

#### ***v) Detection of hybridised probe***

During the probe detection procedure, all washes were performed at room temp with shaking, using clean containers. The composition of buffers used is shown in Table 3.4. Firstly, the membrane was equilibrated in Buffer 1 for 1 min. The membrane was then placed in another dish and blocked in Buffer 2 for 30 min. During this time, the antibody solution was prepared by diluting the anti-DIG-alkaline phosphatase (anti-DIG-AP) to 1:10,000 in Buffer 2 (i.e. 3µl Anti-DIG-AP in 30ml Buffer 2). Buffer 2 was poured off after blocking the blot, and the membrane incubated in the antibody solution for 30 min. The membrane was removed to another clean dish and washed for 2x15 min in Buffer 1. The membrane was then equilibrated in 20ml Buffer 3 for 2 min.

10 ml of colour substrate solution was freshly prepared by mixing 45µl NBT solution and 35µl X-phosphate in 10 ml Buffer 3. After the membrane had equilibrated in Buffer 3 for 1 min, the buffer was poured off and the colour substrate solution applied. Development was allowed to proceed in the dark. Generally, the colour precipitate formed 15-20 min after application of the colour substrate solution, although detection could occur over a much longer period if necessary. After development was complete, the membrane was rinsed with water to prevent further reaction that could lead to a highly coloured background

on the membrane, and the dried membrane sealed in a plastic bag.

### ***Cloning of *B. forsythus* and *P. intermedia* hsp60 genes (PCR products)***

PCR products were cloned into the plasmid vector pCR<sup>®</sup>2.1-TOPO. The plasmid was contained in the TOPO TA Cloning<sup>®</sup> kit (Invitrogen) which provides a one-step cloning procedure for the insertion of PCR products into the plasmid. The technique is very efficient and extremely rapid as no post-PCR procedures are required. The only stipulation is that the PCR products are generated with *Taq* polymerase. *Taq* polymerase causes the products to be synthesised with a single deoxyadenosine (A) overhang at the 3' end of the product, which allows efficient ligation to the overhanging 3' deoxythymidine (T) residues present on the linearised vector.

The kit supplied the plasmid, chemically competent cells (One Shot<sup>™</sup> Competent Cells, which are *E. coli* strain TOP10F' SOC medium and  $\beta$ -mercaptoethanol required for transformation. These and other components used are listed in Table 3.5.

#### ***i) Ligation of PCR products to vector***

For the correct insert:vector ratio required for successful cloning, 4 $\mu$ l of the *B. forsythus* and *P. intermedia* PgN1 C1 PCR products were used (products are approximately 1.6kb in size, and at a concentration of 10ng/ $\mu$ l as determined by visual comparison to standards on agarose gels). The products were added to 1 $\mu$ l pCR<sup>®</sup>-TOPO vector, gently mixed and left to incubate at room temp for 5 minutes. The reaction was briefly centrifuged, the tube placed on ice, and the transformation reaction carried out immediately.



### ***ii) Transformation of competent *E. coli* cells***

A vial of 50µl of OneShot TOP10 competent cells was thawed on ice, and 2µl 0.5M β-mercaptoethanol gently stirred into the cells. 2µl of each cloning reaction (see above) was then mixed into the cells, and the sample incubated on ice for 30 min. The cells were then exposed to heat shock at 42°C for 30 seconds, and then immediately transferred back onto ice, and incubated for a further 2 min. 250µl SOC medium was subsequently added to the reaction tube, and the cells incubated at 37°C for 30 min. Following this, 100µl samples of the cells were evenly spread onto L-agar plates containing 100µg/ml ampicillin, 100mM IPTG and 40µg/ml X-gal. The plates were incubated overnight at 37°C, resulting in the growth of thousands of colonies, from which 10 white colonies were selected for analysis.

### ***iii) Screening of recombinant clones***

Selected recombinant clones (white colonies) were subjected to small-scale plasmid isolation by the boiling method, as described in Chapter 2. The plasmid preps were then analysed by digesting 10µl of mini-prep DNA with *EcoRI* in a total reaction volume of 20µl, as described in Chapter 2. This caused the insert to be excised from the plasmid vector, and the sizes of the inserts determined. This was most easily done by a comparative analysis of the size of the inserts when electrophoresed with the *B. forsythus* hsp60 gene PCR product. 10µl of each digestion reaction was electrophoresed on a 0.8% agarose gel and the insert sizes compared, with the *B. forsythus* hsp60 gene PCR product used as an insert size marker.

Identification of correct hsp60 gene inserts was further verified by Southern blot

analysis. The same electrophoresis gel used to illustrate the sizes of the released vector inserts was transferred onto positively charged membrane and the membrane hybridised to an *E. coli* hsp60-labelled DNA probe as described previously in this chapter. Colorimetric detection of the hybridised probe identified recombinant clones carrying the *B. forsythus* hsp60 gene.

**Table 3.1: PCR buffers and reaction mixes**

*10X reaction buffer*

500mm KCl

100mM Tris-HCl (pH 9.0)

1% Triton X-100

15mM MgCl<sub>2</sub>

*Lower reaction mix*

10X reaction buffer	10μl
dNTPs (10mM stock)	1μl
Taq polymerase (5U/μl stock)	1μl
Molecular biology grade water	38μl

*Upper reaction mix*

Primer PgN1 (1μg/μl stock)	0.2μl
Primer C1 (1μg/μl stock)	0.2μl
Molecular biology grade water	48.6μl

**Table 3.2: Hybridisation stock solutions and buffers**

HCl	250mM hydrochloric acid
Denaturation solution	0.5 M NaOH, 1.5 M NaCl
Neutralisation solution	0.5 M Tris-HCl, pH 7.5; 3M NaCl
20X SSC buffer	3M NaCl, 300mM sodium citrate; pH 7.0
Standard prehybridisation buffer	5X SSC, 1.0% (w/v) Blocking reagent, 0.1% N-lauroylsarcosine, 0.02% sodium dodecyl sulphate (SDS)
2X wash solution	2X SSC containing 0.1% SDS
Blocking reagent	
SDS	10% (w/v) filtered through a 0.2- 0.45µm membrane

**Table 3.3: Random primed DNA labelling solutions**

10X Hexanucleotide mix	62.5 A <sub>260</sub> units/ml random hexanucleotides, 500mM Tris-HCl, 100mM MgCl <sub>2</sub> , 1mM Dithioerythritol (DTE), 2mg/ml BSA; pH 7.2
10X dNTP mix	1mM dATP, 1mM dCTP, 1mM dGTP, 0.65 mM dTTP, 0.35mM DIG-11-dUTP; pH 7.5
Klenow enzyme	2 units/µl DNA polymerase I (Klenow enzyme, large fragment),labelling grade, from <i>E. coli</i> .
EDTA	200mM EDTA, pH8.0
LiCl	4M lithium chloride solution

**Table 3.4: Stock solutions and buffers for colorimetric detection**

Anti-digoxigenin-AP, Fab fragments	Anti-digoxigenin [Fab] conjugated to alkaline phosphatase
NBT solution	75mg/ml nitroblue tetrazolium salt in 70% (v/v) DMF
X-Phosphate solution	50mg/ml 5-bromo-4-chloro-3- idolyly phosphate (X-phosphate) toluidinium salt in 100% DMF
Buffer 1	100mM maleic acid, 150mM NaCl; pH 7.5
Buffer 2	1% (w/v) Blocking reagent dissolved in Buffer 1
Buffer 3	100mM Tris-HCl, pH 9.5; 100mM NaCl, 50mM MgCl <sub>2</sub>

**Table 3.5: TOPO TA cloning and transformation solutions**

SOC medium	
β-mercaptoethanol	
ampicillin	100mg/ml solution, filter sterilised
IPTG	1M solution in water
X-gal	50mg/ml solution in dimethylformamide (DMF)

## **RESULTS**

### ***Derivation of hsp60-specific PCR oligonucleotide primers***

Degenerate oligonucleotide primers for PCR amplification of the hsp60 gene of *B. forsythus* were designed by means of aligning the published DNA sequences of several hsp60 genes and their immediate flanking regions of other bacterial species. From the alignment, a consensus sequence was derived which aided the design of the primers

The sequences of the consensus primers selected were:

5' ATTATGGCAAAGAAATCAAATTCG 3' (PgN1) and

5' TTACATCATGCCGCCCA 3' (C1).

These primers amplify the entire coding region of the *groEL* component of the hsp60 gene of *B. forsythus* and *P. intermedia*, but were not specific for *T. denticola*, *P. nigrescens* or *S. mutans*. Primer PgN1 annealed at the start site of the open reading frame (ORF) of the hsp60 gene (the second triplet of the primer, ATG, annealing to the start codon of the gene). Primer C1 annealed at the end of the ORF (the first triplet of this primer, TTA, annealing to the stop codon, AAT, of the gene).

### ***Analysis of hsp60 PCR products***

#### ***i) Size analysis***

PCR of *B. forsythus* and *P. intermedia* chromosomal DNA with the consensus primers yielded a discrete band of approximately 1.8-kb (Fig 3.1 and Fig 3.2) which was of the expected size. No products of similar size were generated using genomic DNA from *P. nigrescens*, *T. denticola* or *S. mutans* (Fig 3.2).

## ***ii) Southern blot analysis***

To unequivocally identify the PCR product as the hsp60 gene of *B. forsythus*, the product was subjected to Southern blot analysis. The product was blotted with a probe derived from the hsp60 protein of *E. coli*. The probe clearly hybridised to the PCR product (Fig 3.3) under relaxed washing conditions (2xSSC/0.1% SDS at 68°C).

## ***Analysis of recombinant clones***

### ***i) Restriction endonuclease digestion of mini prep DNA***

The hsp60 PCR products of *B. forsythus* and *P. intermedia* were cloned into the plasmid vector pCR 2.1-TOPO, which has been designed with 3' T overhangs that complement the A overhangs present on PCR products synthesised by *Taq* DNA polymerase. These complementary sequences allow for a simple and quick cloning protocol. A plasmid map of vector pCR2.1-TOPO is shown in Fig 3.4. *E. coli* strain TOP10F' was transformed with the ligated vector, and recombinant clones identified by their white colour.

*EcoRI* cleavage sites flanking the insertion site of the vector allow restriction endonuclease analysis of selected clones. Digestion of the plasmid with *EcoRI* releases the DNA fragment that has been inserted into the cloning site of the vector.

For all *B. forsythus* clones analysed, a 1.8-kb insert was released, which confirmed successful cloning of the *B. forsythus* groEL gene (Fig 3.5). A lower rate of recombination success was achieved for the *P. intermedia* hsp60 gene with three clones out of 12 analysed apparently carrying the gene (Fig 3.6).

## ***ii) Southern blot analysis***

To verify the identity of the 1.8-kb insert carried by the transformants, *B. forsythus* fragments released by digestion with *EcoRI* were Southern blotted using a DIG-labelled *E. coli* hsp60 probe. As can be seen in Fig 3.7, the *B. forsythus* inserts all hybridised to the probe, thus confirming that the successfully cloned insert was the hsp60 gene of *B. forsythus*.



## DISCUSSION

The *groEL* portion of the *hsp60* gene of *B. forsythus* has been successfully identified and isolated via PCR, using consensus primers. The PCR product, a 1.8-kb fragment encoding the *groEL* gene, was cloned into pCR2.1-TOPO, which is a plasmid vector specifically designed for simple and rapid cloning of PCR products. *E. coli* strain TOP10F' was then transformed with the ligated plasmid. The *hsp60* gene of *P. intermedia* was similarly identified and cloned into pCR2.1-TOPO to facilitate later DNA sequencing of the gene.

This approach to the detection and cloning of homologous genes in other species has been frequently utilised previously. Nakano *et al* (1995) used *E. coli*-derived primers to amplify a portion of the *groEL* gene of the major periodontal pathogen, *A. actinomycetemcomitans*. These primers amplified a fragment which was then labelled with digoxigenin-dUTP and used to probe Southern blots of restriction endonuclease digests of *A. actinomycetemcomitans* chromosomal DNA. The probe detected 3-kb fragments which, upon analysis, were found to contain the entire *groESL* operon of *A. actinomycetemcomitans* (Maeda *et al*, 1994). The *hsp60* gene of *Chlamydia trachomatis* serovar E was amplified by PCR using primers derived from the DNA sequence of serovar A of the same species. The product was gel-purified and digested with *Sma*I prior to being ligated into a plasmid vector digested to provide blunt-ends (Horner *et al*, 1996). A similar system was employed to isolate the *hsp60* genes of various *Neisseria* species (Pannekoek *et al*, 1995). Whilst this methodology produced the desired results, there are several intervening procedures between PCR and eventual cloning. The pCR2.1-TOPO vector used in this chapter for cloning of the *B. forsythus* and *P. intermedia* *groEL* genes possesses the distinct

advantage of being able to ligate PCR products directly, unmodified and uncleaned. As an alternative method for identifying hsp60 genes, another commonly employed technique is to digest the chromosomal DNA of the species in question with various endonucleases to produce a range of DNA fragments of different sizes. These fragments are then hybridised to a hsp60 probe using the Southern blot method. A fragment to which the probe hybridises can then be gel purified, digested and ligated into a suitably prepared vector. This technique has led to the successful identification and cloning of the hsp60 genes of *Bacillus subtilis* (Schmidt *et al*, 1992), *Clostridium acetobutylicum* (Naberhaus and Bahl, 1992) and the periodontal pathogen *P. gingivalis* (Maeda *et al*, 1994).

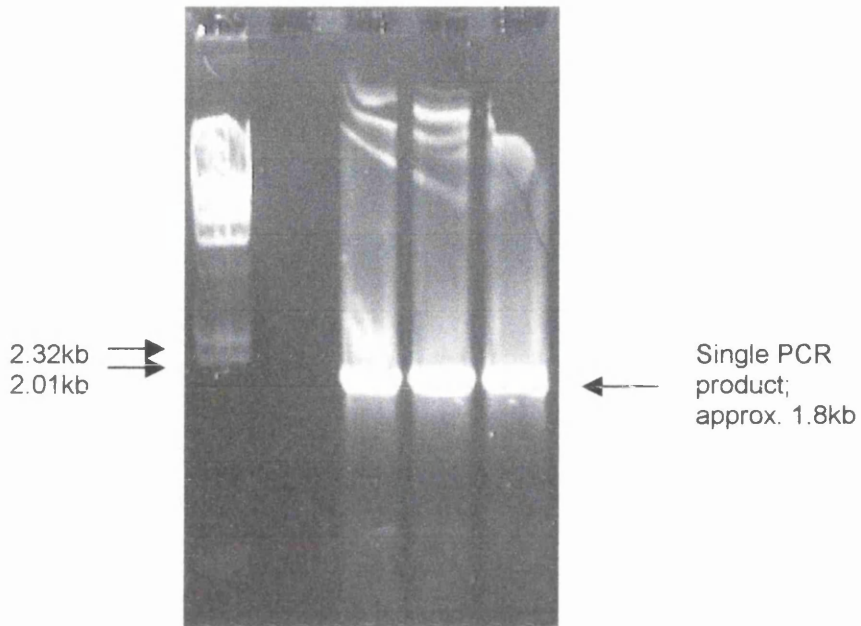
Another popular approach for identifying desired genes involves the construction of a chromosomal gene library in plasmid or bacteriophage vectors. The chromosomal gene library is formed by partially digesting the bacterial chromosomal DNA with *Sau3A*, and cloning the fragments into a vector such as pUC18. The cloned fragments are then screened by blotting with a genomic or protein probe specific for hsp60, and reactive clones isolated from the library. This method has been well applied to enable the cloning of the hsp60 genes of *Chlamydia psittaci* (Morrison *et al*, 1989) and *B. subtilis* (Li and Wong, 1992).

In summary, this chapter describes the design of degenerate oligonucleotide primers for the PCR amplification of the groEL fragment of the hsp60 gene of bacterial species. The primers successfully amplified the hsp60 genes of *B. forsythus* and *P. intermedia*, but were found not to amplify the corresponding gene from *T. denticola*, *P. nigrescens* or *S. mutans*. The amplified hsp60 genes

were cloned into the vector pCR<sup>®</sup>2.1-TOPO, and the cloned genes then subjected to DNA sequence analysis as described in Chapter 4.

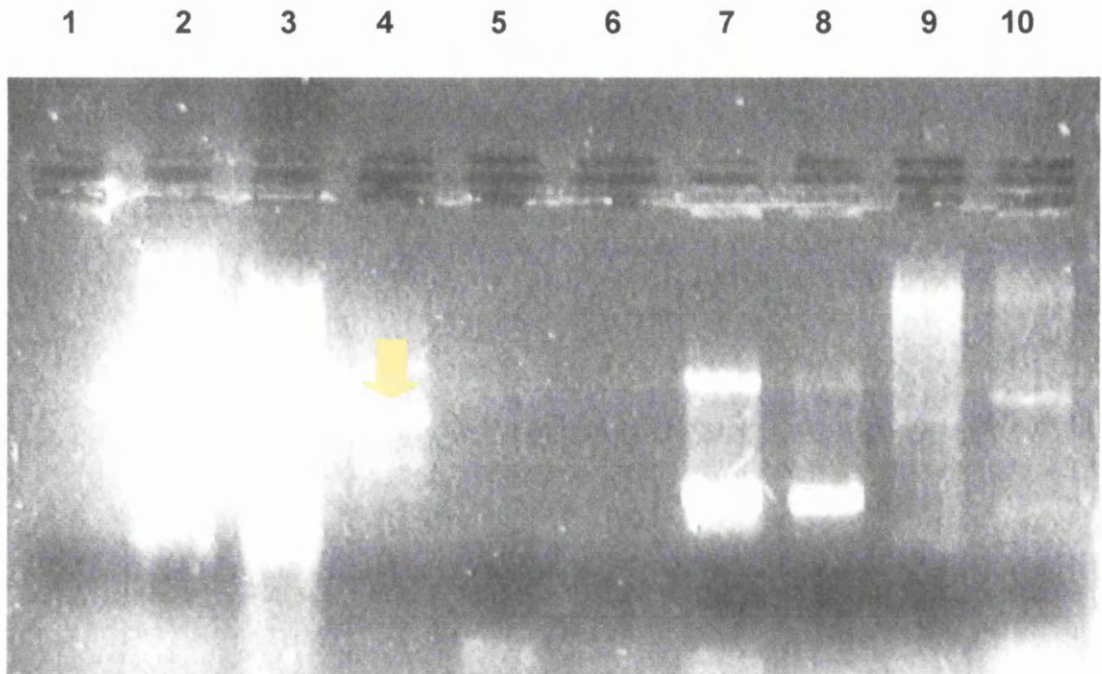
### FIGURE 3.1

PCR amplification of the *B. forsythus* hsp60 gene using degenerate primers



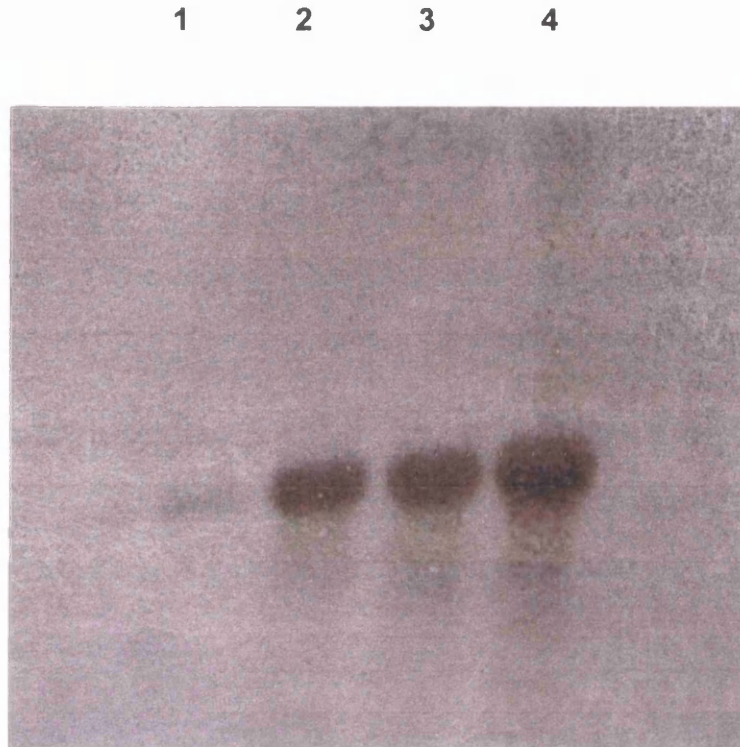
**Figure 3.1** PCR amplification of the *B. forsythus* hsp60 gene using degenerate primers PgN1 and C1. *Hind*III digested  $\lambda$  DNA fragments were used as size markers (23.1, 9.42, 6.56, 4.36, 2.32, 2.01kb) (lane 1), negative control (lane 2), *B. forsythus* samples (lanes 3-5).

**FIGURE 3.2.** PCR amplification of hsp60 genes from various periodontal micro-organisms.



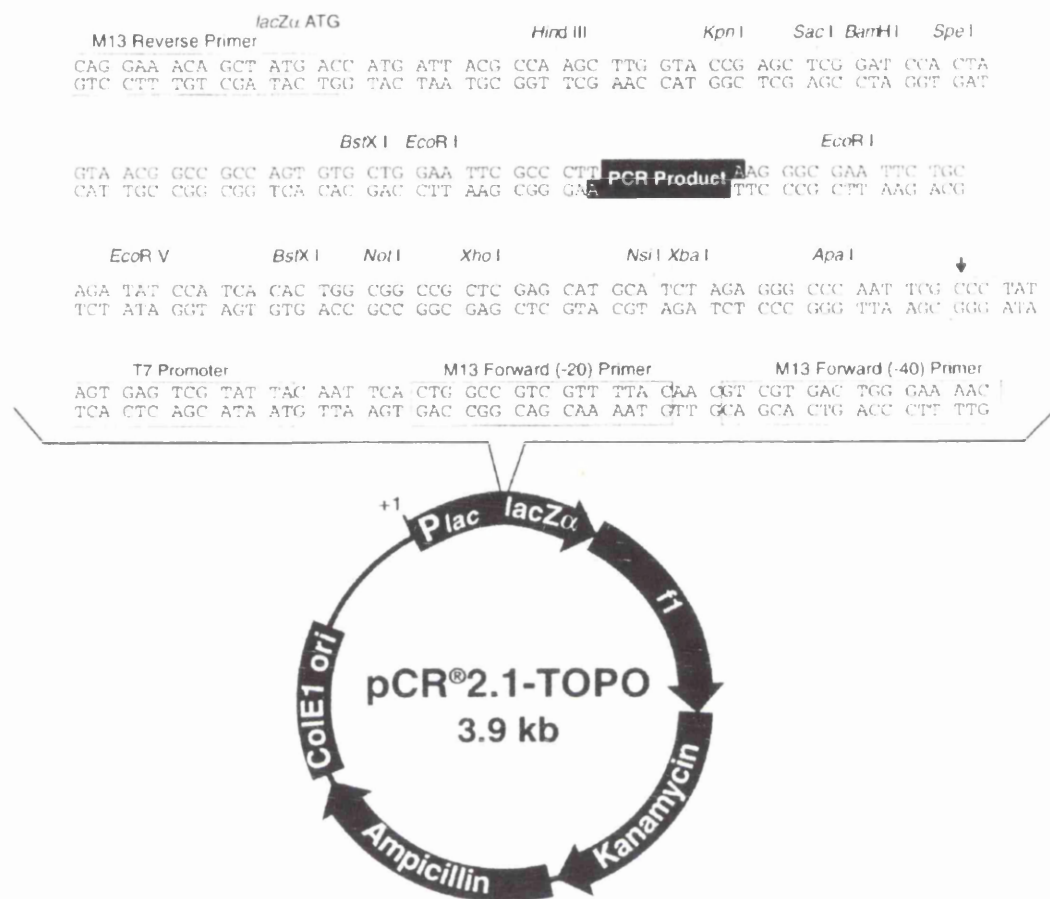
**Figure 3.2.** Degenerate oligonucleotide primers PgN1 and C1 were used to amplify the hsp60 genes from various periodontal micro-organisms. A negative PCR control was electrophoresed (lane 1), along with products generated from *B. forsythus* (lane 2), *P. gingivalis* (lane 3), *P. intermedia* (lane 4), *T. denticola* (lanes 5 and 6), *P. nigrescens* (lanes 7 and 8), *S. oralis* (lane 9) and *S. mitis* (lane 10). A product of the same size as the positive controls in lanes 2 and 3 was generated using *P. intermedia* template DNA (arrow), and was further investigated to characterise the hsp60 of this organism. PCR products were also generated from *P. nigrescens* and Streptococci templates (lanes 7-10) but were too small to carry the entire hsp60 gene. These products were not studied further, although they may have been carrying fractions of the gene.

**FIGURE 3.3.** Southern blot analysis of *B. forsythus* hsp60 PCR products using an *E. coli* hsp60 probe.



**Figure 3.3.** Southern blot analysis of *P. gingivalis* and *B. forsythus* hsp60 PCR products. PCR products generated using primers PgN1 and C1 were electrophoresed and transferred to hybridisation membranes. Transferred DNA was probed with DIG labelled *E. coli* hsp60 DNA (whole groEL gene) previously amplified with primers N1 and C1. Lane 1= *P. gingivalis* PCR product, a positive control for the PCR reactions. Lanes 2-4 are *B. forsythus* PCR products, which appear to have a higher affinity for the *E. coli* probe than the *P. gingivalis* product had. Primers PgN1 and C1 were known to amplify the entire hsp60 gene of *P. gingivalis*, indicating that the *B. forsythus* product was the correct size to be carrying the hsp60 gene.

**FIGURE 3.4. Physical map and cloning site of plasmid pCR®2.1-TOPO.**

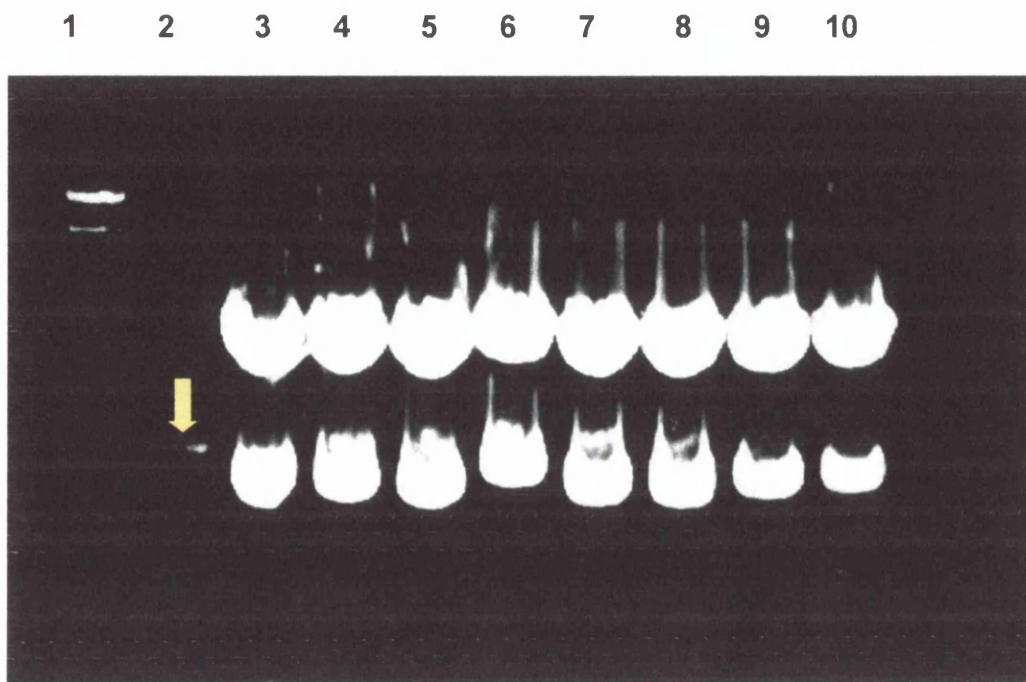


**Figure 3.4.** Physical map and cloning site of the plasmid pCR®2.1-TOPO. The map shows features of pCR®2.1-TOPO and the sequence surrounding the cloning site. The insertion and ligation point for *Taq* polymerase amplified PCR products is indicated, as are primer recognition sites.

Plasmid map Copyright 1997 Invitrogen Corporation, Carlsbad, CA, USA. All rights reserved. Used by permission.



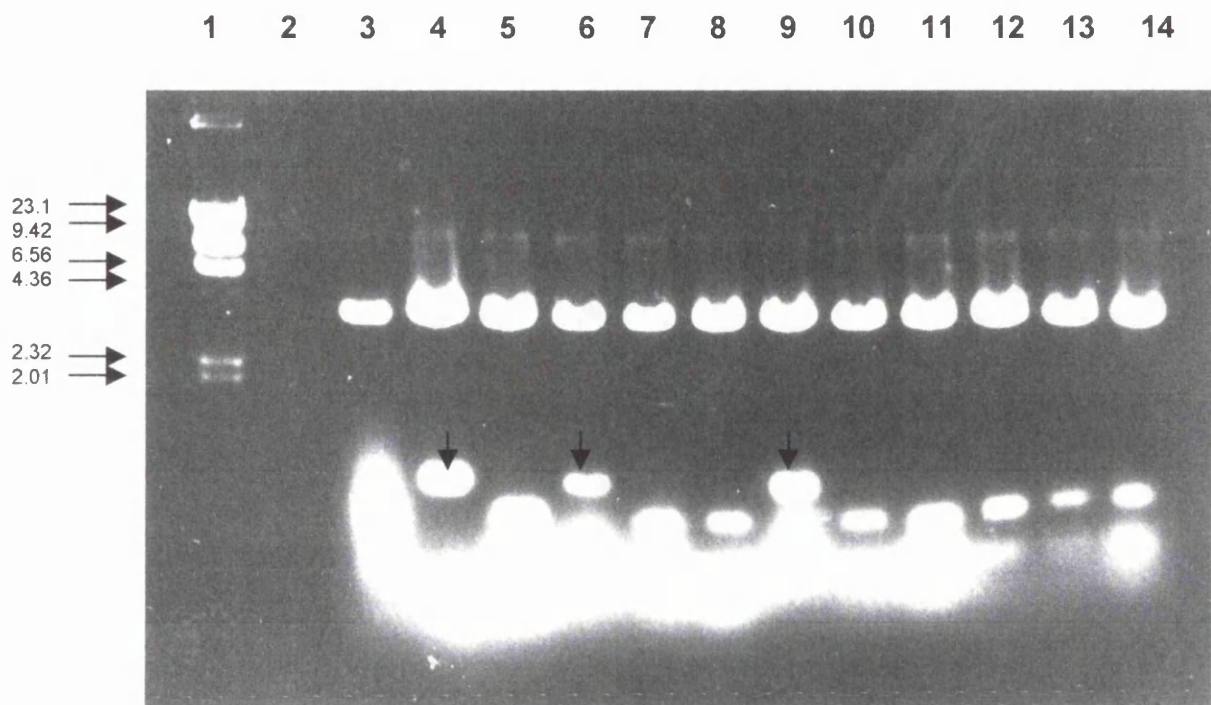
**FIGURE 3.5.** Mini-prep analysis of plasmid constructs pBf1.8.



**Figure 3.5.** Mini-prep analysis of plasmid pBf1.8. Eight recombinant clones were randomly selected and plasmid purified using the small scale boiling method. Plasmid DNA was digested with *Eco*RI (lanes 3-10). *Hind*III digested  $\lambda$  DNA fragments were used as size markers (23.1, 9.42kb bands apparent, lane 1). Lane 2 is the *B. forsythus* hsp60 PCR product (1.8kb, yellow arrow), which indicates that the digest-released cloning fragments are of the correct size

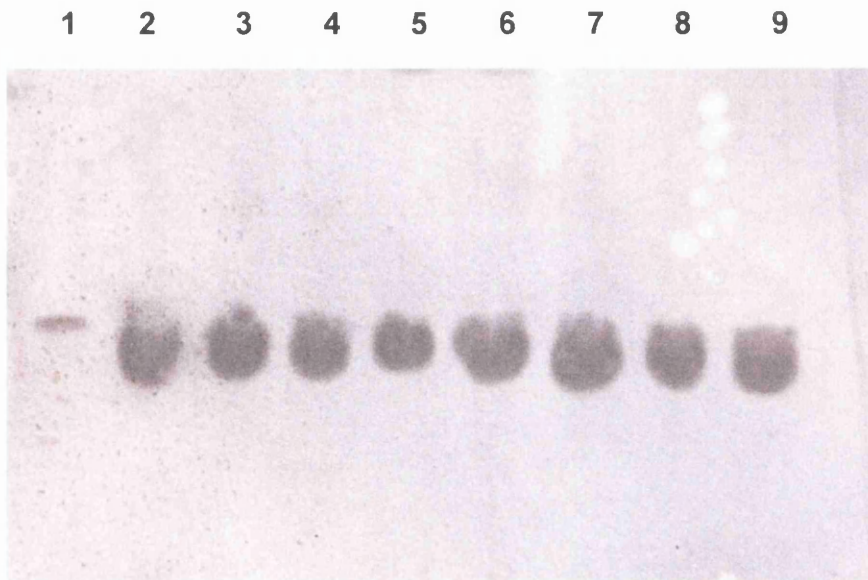


FIGURE 3.6. Mini-prep analysis of plasmid constructs pPi1.6.



**Figure 3.6.** Mini-prep analysis of plasmid pPi1.6. 12 recombinant clones were randomly selected and plasmid purified using the small scale boiling method. Plasmid DNA was digested with *Eco*RI and released DNA inserts electrophoresed. *Hind*III digested  $\lambda$  DNA fragments were used as size markers (lane 1, sizes in kb) and digested plasmid loaded in lanes 3-14. Recombinant clones carrying a DNA insert of the correct estimated size of the *P. intermedia* hsp60 gene are indicated in lanes 4, 6 and 9 (black arrows). The pCR2.1 TOPO plasmid is 3.9kb, seen in lanes 3-14.

**FIGURE 3.7.** Southern blot analysis of plasmid pBf1.8 mini-preps.



**Figure 3.7.** Southern blot analysis of pBf1.8 mini-preps (gel shown in Fig 3.5). DNA inserts in plasmid pBf1.8 released by endonuclease digestion were transferred to hybridisation membrane and probed with DIG labelled *E. coli* hsp60 DNA (lanes 2-9). Lane 1 is a PCR positive control.

## **CHAPTER 4**

**Determination of the DNA sequence of the *B. forsythus* hsp60 gene and analysis of the predicted amino acid sequence, and partial sequence of the *P. intermedia* hsp60 gene**

## **INTRODUCTION**

The hsp60s have become the focus of intense research since the realisation that hsp60 is a 'common antigen' expressed by a wide variety of bacterial species, many of which are human pathogens (Thole *et al*, 1985; Young *et al*, 1987; Shinnick, 1991; Jones *et al*, 1993). Not only is hsp60 common to bacteria, but highly conserved homologues have been identified in yeasts and higher eukaryotic cells, including a human counterpart, mitochondrial matrix protein P1 (Dudani and Gupta, 1989; Jindal *et al*, 1989). More distantly related homologues, which still maintain functional and sequence similarity, are the Tcp-1 (t-complex polypeptide 1) family of proteins found in archaeobacteria and eukaryotic cell cytosol (Gupta, 1995).

The burgeoning interest in hsp60 has lead to numerous groups sequencing hsp60 genes from across the entire spectrum of prokaryotic and eukaryotic species (reviewed in Shinnick, 1991; Zeilstra-Ryalls, 1991; Gupta, 1995). These studies have revealed that the functional conservation of hsp60s is reflected at the genomic level. The level of sequence homology maintained between all species is remarkable and virtually unprecedented in the living kingdom.

Measuring sequence similarity as the number of positions at which identical amino acid residues can be found in a pairwise alignment, the level of homology between different genera ranges from the lowest level at approximately 45% to over 80%. Even between organisms as distantly related as humans and *E. coli*, the level of hsp60 sequence conservation is 50%. Of the amino acids that are not identical, up to 20% of the substitutions are conservative replacements (Gupta, 1995). Such conservation indicates that these proteins express a universally important function.

Hsp60 genes continue to be isolated and sequenced. Whilst this may appear superfluous considering the well documented high level of genomic homology between species, the need to further characterise hsp60 genes of more species is a very real one, which will allow an accurate awareness of their functional and immunogenic importance to be founded.

Recent publications have brought to light the fact that some disparate proteins have been found to have genetic homology with hsp60. These include a protein of *A. actinomycetemcomitans* that possesses potent osteolytic activity, demonstrating bone resorbing activity at ~1pM molar concentrations. The structure of this protein was also examined by electron microscopy and was found to form the double ring structure characteristic of hsp60 homologues (Kirby *et al*, 1995). These findings suggest that there are aspects of hsp60s that have yet to be examined, and a better understanding of the protein will only result from further studies of hsp60, be it at a molecular, structural or functional level.

As mentioned previously, hsp60 of bacterial origin has been demonstrated to elicit a cross-reactive immune response (Young *et al*, 1987; Thole *et al*, 1988; Winfield, 1991; Kaufmann and Schoel, 1994; Handley *et al*, 1995; Krenn *et al*, 1996; Hinode *et al*, 1998), that may provoke an autoimmune reaction in humans. Those who expound this theory as applied to periodontal disease have sequenced the hsp60 genes of the major periodontal pathogens *A. actinomycetemcomitans* and *P. gingivalis* to allow further immunologic studies of the protein (Maeda *et al*, 1994; Nakano *et al*, 1995; Hinode *et al*, 1998). An understanding of the DNA and amino acid sequence of the proteins is essential to determine the level of sequence homology shared between species, and thus

their potential to raise a cross-reactive immune response. Elucidating the entire DNA and amino acid sequence of a protein is an important first step towards characterising individual epitopes and designating them as unique species-specific epitopes or as well-conserved ones that may result in cross-reactive antibodies, and can also allow identification of immunodominant epitopes. This chapter presents the DNA and predicted amino acid sequence of the hsp60 gene of *B. forsythus*, and sequence data for the 3' region of the hsp60 gene of *Prevotella intermedia*. This is the first report of the nucleotide sequence of the *B. forsythus* hsp60 gene. Alignments of these gene sequences with the published sequences derived from other species shows that the genes are characteristically well conserved.

## **MATERIALS AND METHODS**

### ***DNA sequencing: manual method***

Double-stranded DNA was sequenced using the dideoxy chain termination method. Single stranded DNA templates were produced from plasmid DNA by alkaline denaturation. DNA to be sequenced was cloned into pCR2.1-TOPO™ as previously described. The gene was completely sequenced on both strands using a genome walking method to generate sequence data.

### ***i) Sequencing primers***

Commercially available primers (M13 and Reverse) and custom-made hsp60 specific primers (Cruachem) were diluted to 5ng/μl and stored at -20°C until required.

### ***ii) Primer design***

As a general rule the primer sequence contained:

1. no inverted repeats
2. no direct repetition
3. a G/C content of 40-60%
4. no primer-dimer formations
5. a G/C 3' tail, of no more than 3 G or C residues.

The absence of inverted repeats ensures that the primer is less able to form hair-pin structures by self-annealing. Direct repeats may cause the primer to anneal in the wrong position to the template DNA. The correct proportion of GC residues increases the primer-template binding strength, and a GC domain at

the 3' tail ensures a strong bond of the primer to the template at the crucial end of the primer, from whence elongation will proceed.

### ***iii) Radioactive label***

The radioactive label used for manual sequencing was deoxyadenosine 5-[ $\alpha$ - $^{35}\text{S}$ ] thiotriphosphate triethylammonium salt (dATP, Sp isomer) (Amersham).

The label had a specific activity of >1200 Ci/mmol and a concentration of 10mCi/ml.

### ***iv) Denaturation reaction***

Generally, for every 3 kb of plasmid, 2 $\mu\text{g}$  of DNA was used as template. DNA was denatured with NaOH at 0.4M, and H<sub>2</sub>O added to a final reaction volume of 10 $\mu\text{l}$ . The sample was mixed by pipetting and incubated at room temp for 10 min. The reaction was then neutralised by addition of 3 $\mu\text{l}$  3M NaOAc (pH 5.3), and dH<sub>2</sub>O added to a final volume of 20 $\mu\text{l}$ . DNA was precipitated with 3 volumes 100% ethanol and incubation at -70°C for 30 min prior to centrifugation at 14,000 rpm for 10 min. The isolated DNA pellet was washed with 70% ethanol and centrifuged again, and the resulting pellet lyophilised before being resuspended in 10 $\mu\text{l}$  dH<sub>2</sub>O.

### ***v) Annealing reaction***

To 10 $\mu\text{l}$  of denatured DNA, 2 $\mu\text{l}$  annealing buffer (Table 4.1) and 2 $\mu\text{l}$  sequencing primers at 5ng/ $\mu\text{l}$  was added. The reaction was thoroughly mixed and incubated at 37°C for 20 min, and then at room temp for 10min.



#### ***vi) Labelling reaction***

An enzyme pre-mix containing the following was formed on ice: 1µl dH<sub>2</sub>O, 3µl labelling mix, 2µl T7 DNA polymerase diluted 1:4 in dilution buffer, and 1µl <sup>35</sup>S-dATP. This was briefly centrifuged to mix, and 6µl of the premix added to the DNA sample with primers annealed (i.e. the product of the annealing reaction). The sample was thoroughly mixed and incubated at room temp for 5 min. 2.5µl of A, C, G and T mixes (Table 4.1) was added to appropriately labelled tubes and warmed at 37°C for 2 min. The concentration of dNTPs and ddNTPS in the reaction mixes (supplied in the kit) are balanced such that there is an equal chance of a ddNTP being incorporated at each position within the length of the sequence, terminating the extension of that fragment. 4.5µl of the DNA/label/enzyme mix was added to each A, C, G and T tube and mixed by brief centrifugation. The tubes were incubated at room temp for 5 min, and reactions then terminated by adding 5µl stop solution (Table 4.1) per tube. The reactions were stored at -70°C until required. Immediately prior to loading on the sequencing gel, the samples were heated at 80°C for 3 min. Half of each A, C, G and T sample was loaded for a “long run” sequence read, and the other half of each mix loaded approximately 3 hours later for a short run read.

#### ***vii) Denaturing polyacrylamide sequencing gel***

A 6% acrylamide gel was prepared using the solutions listed in Table 4.2, and wedge shaped gels poured. The MacroPhor gel sequencing system (Pharmacia) was used according to manufacturer's instructions. Prior to pouring the gel, the notched glass plate was treated with Bind-Silane solution (Table 4.2) to adhere the gel to this plate. The upper thermostatic plate was polished

twice with Repel-Silane to ensure that the gel bonded to the notched glass plate separated easily from the upper plate following electrophoresis.

Gels were cast on a gradient for ease of gel pouring, using a platform placed at an angle to support the glass plate sandwich. The glass plates were clamped together at the bottom end away from the upper notch to give a wedge-shaped gel. The well-forming comb was inserted, the gel placed horizontally and left to polymerise for at least 1 hour.

Prior to loading samples, gels were pre-electrophoresed at 2000V and 60°C for 30 min to remove charged contaminants from the gel. Before the samples were loaded, the wells were flushed out with buffer to remove air bubbles and any urea deposits or debris in the wells. 6µl of sample was loaded per well, and electrophoresis performed at 2000V. When the bromophenol blue dye front reached the bottom of the gel (after ~3 hours) the remaining half of the samples were loaded and electrophoresis proceeded until this dye front also reached the bottom of the gel. This resulted in a “long run” and a “short run” which yielded a maximum amount of sequence data. After completion of electrophoresis, the upper thermostatic plate was prised apart from the gel and the gel, still bonded to the notched glass plate, soaked in 10% acetic acid for 15 min to remove urea. The gel was then dried with a hair drier and wrapped in cling film. The gel was exposed to X-ray film in a light-proof box overnight, prior to development.

#### ***viii) DNA sequence data analysis***

Sequence data was obtained from developed autoradiograms. Data was initially compiled using the University of Wisconsin's GCG package of programs, and

the collated sequence ultimately submitted to EMBL (accession number AJ006516).

### ***DNA sequencing: automated method***

Genes inserted into the plasmid vectors pCR2.1-TOPO™ were sequenced on an automated DNA sequencer (LI-COR DNA sequencer 4200 series), using a cycle sequencing method. The Amersham Thermo Sequenase labelled primer cycle sequencing kit with 7-deaza-dGTP was used.

#### ***i) Sequencing primers***

Fluorescently labelled primers were synthesised by MWG-Biotech.

Commercially available primers M13 Forward and M13 Reverse were used, which target the plasmid sequence flanking the insertion site of the vector.

Custom synthesised hsp60-specific primers were also designed, following the same guidelines as for the manual sequencing primers. 2pmol of primer was used per reaction.

#### ***ii) Plasmid DNA template preparation***

Plasmid DNA to be sequenced was isolated from overnight cultures of bacteria using the Wizard® Plus Minipreps DNA purification system (Promega), as described in Chapter 2. The concentration of the purified DNA was crucial for good sequencing reactions to occur. For optimal results, 130ng/kb plasmid DNA was required per reaction, and ideally this should be in a volume not greater than 5µl to decrease the chance of introducing contaminants into the reaction.

### ***iii) Cycle sequencing reactions***

Pre-labelled primers were purchased from MWG Biotech and used to generate amplification products via cycles of thermal denaturation, annealing and extension/termination using Thermo Sequenase heat stable DNA polymerase. The reaction mixes were performed on ice before being transferred to a thermal cycler. A DNA/primer pre-mix was first formed as below:

plasmid template	<5µl, using 130ng DNA per kb template
labelled primer, 2pmol	<5µl
DMSO	0.7µl
dH <sub>2</sub> O	to 21µl

1.5µl aliquots of the A, C, G and T reagents, contained within the kit, were each transferred into 1.5ml centrifuge tubes. 4.5µl of the DNA/primer pre mix was aliquoted into each tube, and overlayed with 1 drop of Chill-out 14™ liquid wax (MWG Biotech; the liquid wax solidifies below 10°C and can be pierced with a micro-pipette tip to remove the aqueous phase of chilled samples). Cycling reactions were then performed in a Perkin Elmer 420 thermal cycler as follows: 95°C, 30 sec (1 cycle): 95°C 10 sec; Ta°C 30 sec; 70°C 30 sec (20 cycles): 95°C 10 sec; 70°C 30 sec (15 cycles).

On completion of the cycling reactions, 6µl of stop solution was added to each tube. 1.5µl of each reaction was loaded onto a denaturing polyacrylamide gel.

### ***iv) Preparation of denaturing polyacrylamide sequencing gels.***

Gels were formed by pouring liquid acrylamide solution between 2 glass plates (41 cm), and then allowing this solution to polymerise prior to electrophoresis.

Both glass plates were thoroughly cleaned before use by subsequent washings with 0.1M HCl, 0.1M NaOH, distilled water and finally 100% ethanol, using lint-free tissue. The gel sandwich was formed by assembling spacers between the glass plates, with the glass plates placed with the bevelled edges together for ease of separation later. The plates were fixed together with rail clamps.

Subsequently, the gel solution was prepared as described in Table 4.3. Prior to the addition of the polymerising agents TEMED and APS, the gel solution was de-gassed for 20 min using a vacuum pump.

The glass plate sandwich was laid horizontally, and the top end lifted 1 inch to give a gradient for ease of casting. The prepared gel mix was applied with a 50ml syringe to the well at the top end of the plate sandwich. The surface of the plates was firmly tapped whilst the gel was being applied to prevent air bubbles from being trapped within the gel. Once the gel had been poured, the gel sandwich was laid flat and the well-forming spacer inserted between the top edges of the two plates. The gel was left to polymerise for at least one hour.

#### ***v) Pre-electrophoresis***

The polymerised gel was installed in the LI-COR sequencer according to manufacturer's instructions, and the upper and lower buffer tanks filled with 1x long run TBE. To remove contaminants from the gel and to allow focusing of the laser, the gel was pre-electrophoresed at 1500V and 50°C for 30 min, and the system prepared for scanning as according to the manufacturer.

#### ***vi) Loading and running the gel***

Following electrophoresis, the well-forming spacer was carefully removed from

the gel and the well flushed out with a syringe to remove acrylamide debris and excess urea. After the well had been cleared, a 64-lane sharktooth comb was inserted such that the tip of the teeth touched the top edge of the gel. Sample wells were formed by the spaces that existed between the comb teeth.

The gel was run at 1500V and 50°C until such time that the reactions had run to completion and could no longer be read. This was after approximately 8 hours.

#### ***vii) Analysis of sequence data***

Sequence data was automatically read by BasemagIR™ Software (for LI-COR DNA sequencers). Generally, up to 800 bases could be read per reaction. The software package stopped reading data after this time, when the number of ambiguities or unreadable regions of sequence increased.

**Table 4.1: Manual DNA sequencing reaction buffers and solutions**

*10X Annealing buffer*

100mM Tris HCl (pH 8.0)

50mM MgCl<sub>2</sub>

*ddG Termination mix*

80μM dGTP, 80μM dATP, 80μM dCTP, 80μM dTTP, 8μM ddGTP, 50mM NaCl

*ddA Termination mix*

80μM dGTP, 80μM dATP, 80μM dCTP, 80μM dTTP, 8μM ddATP, 50mM NaCl

*ddT Termination mix*

80μM dGTP, 80μM dATP, 80μM dCTP, 80μM dTTP, 8μM ddTTP, 50mM NaCl

*ddC Termination mix*

80μM dGTP, 80μM dATP, 80μM dCTP, 80μM dTTP, 8μM ddCTP, 50mM NaCl

*Stop solution*

95% formamide

20mM EDTA

0.05% Bromophenol blue

0.05% Xylene Cyanol FF

**Table 4.2: Manual DNA sequencing and gel mixes and solutions**

*Bind-Silane solution*

100% ethanol	20ml
10% (v/v) acetic acid	5ml
Bind-Silane	75µl

*Gel mix using LONG RANGER*

urea	31.5g
50% stock LONG RANGER (Flowgen)	9.0ml
10X TBE	7.5ml
dH <sub>2</sub> O	To 75 ml

Prior to use add 0.6 ml 10% APS and 56µl TEMED.

*10% ammonium persulphate (APS)*

ammonium persulphate	0.1g
dH <sub>2</sub> O	1ml



**Table 4.3: Automated DNA sequencing stock solutions and gel mixes**

*6% Sequencing gel mix*

Rapid gel XL solution, 40% stock (Amersham)	7.5ml
Urea	21g
10xTBE long run	5ml
H <sub>2</sub> O	28ml
DMSO	500µl
TEMED	50µl
10% APS, freshly prepared	350µl

APS was made as required, by dissolving 0.1g APS in 1ml MB grade H<sub>2</sub>O.

The reagents minus TEMED and APS were stirred together until the urea dissolved. The mixture was then de-gassed for 20 min using a vacuum pump, prior to the addition of the polymerising agents TEMED and APS. The solution was kept on ice to slow down the polymerisation process whilst the gel was being poured.

**Table 4.3 (continued)**

*10x TBE Long Run Buffer*

Tris base	162g
Boric acid	27.5g
EDTA	9.3g
Molecular biology grade H <sub>2</sub> O	To 1 litre

The dry ingredients were weighed out and placed in a flask. 900ml MB grade water was added, and the solution stirred on a heated plate until all the components had dissolved. The pH of the solution was measured and adjusted to pH 8.5 with NaOH. The volume was then made up to 1 litre with MB grade water.

## **RESULTS**

### ***Molecular cloning of the B. forsythus hsp60 gene***

A 1.8-kb PCR product was inserted into the cloning site of the plasmid vector pCR2.1-TOPO as described in Chapter 3. The resulting recombinant plasmid was designated pBf1.8.

### ***Determination of the DNA sequence of the B. forsythus hsp60 gene***

To initiate sequencing of the DNA insert of pBf1.8 commercially available primers M13 Forward and M13 Reverse were used. Thereafter, *B. forsythus* hsp60-specific custom-made oligonucleotide primers were used to rapidly generate sequence data using a genome walking method. The cloned PCR product was completely sequenced on both strands, so that each nucleotide was sequenced at least twice. An autoradiograph obtained from a typical manual sequencing gel is shown in Fig 4.1. A sample of data resulting from BasemagIR<sup>®</sup> sequence analysis of an automated sequencing reaction is shown in Fig 4.2.

### ***DNA sequence alignment of the hsp60 genes of B. forsythus and P. gingivalis***

The DNA sequence obtained from the hsp60 gene (groELunit) of *B. forsythus* represents 1635 nucleotides and is shown in Fig 4.3. An alignment of this nucleotide sequence with the sequence from the corresponding gene of the closely related species *P. gingivalis* is shown in Fig 4.4. The *B. forsythus* hsp60 nucleotide sequence shares 73% identity with the *P. gingivalis* hsp60 sequence (identical at 1192 out of 1635 residues).

### ***Analysis of the predicted amino acid sequence of B. forsythus hsp60***

Translation of the DNA sequence of the hsp60 gene (groEL unit) from *B. forsythus* in all six reading frames identified only one open reading frame of sufficient length to code for a protein of the size expected for hsp60. The DNA sequence of the gene and the predicted amino acid sequence of the *B. forsythus* hsp60 protein are shown in Fig 4.3. The *B. forsythus* groEL gene has an ATG initiation codon at position 1, and a chain termination codon at position 544 of the amino acid sequence. The predicted translation product is a 544 amino acid, 58-kDa protein. This compares with a 545 amino acid (58.4-kDa) groEL protein from *P. gingivalis* and a 547 amino acid (57-kDa predicted molecular weight) predicted gene product from the hsp60 gene of *A. actinomycetemcomitans*.

### ***Comparison of the predicted B. forsythus hsp60 amino acid sequence with those from other species***

An alignment of the *B. forsythus* hsp60 amino acid sequence and the hsp60 amino acid sequences from *A. actinomycetemcomitans*, *P. gingivalis*, *E. coli* and human mitochondrial protein P1 is shown in Fig 4.5. The genes possess a notably high degree of homology, which is characteristic of the hsp60 family of proteins. The highest level of sequence identity is shared between the hsp60 genes of *B. forsythus* and *P. gingivalis*, which is predictable as both organisms belong to the same *Bacteroides* taxonomic group.

From the alignment, it can be calculated that the *B. forsythus* groEL amino acid sequence shares 81% sequence identity with the hsp60 gene of *P. gingivalis*,

63% identity with *A. actinomycetemcomitans*, 62% identity with *E. coli* and 50% identity with the human homologue, mitochondrial protein P1. Regions of amino acid sequence which were common to all species are identified in the consensus sequence shown in Figure 4.4.

### ***Predicted secondary structures and features of the B. forsythus hsp60 amino acid sequence***

From the amino acid sequence derived for *B. forsythus* hsp60, a prediction of some of the protein's secondary structures could be made via use of the PEPLOT and PEPTIDESTRUCTURE functions of the Genetics Computer Group Wisconsin package (Devereux *et al*, 1984) (Fig 4.6). These programmes illustrate various measures of protein secondary structure on parallel lanes of one co-ordinated plot i.e. the amino acid sequence. The structural predictions are based on the average, sum or product of features of a specific residue contained within a 7-residue window. Thus some residue properties are dependent on other residues within an immediate proximity.

The plot measures the propensity for any residue to form  $\alpha$  helices and  $\beta$  sheets (according to parameters defined by Chou and Fasman, 1978; and Garnier *et al*, 1978), displayed diagrammatically in lanes (e) and (f).  $\alpha$  helices appear to be the dominant secondary structure of the protein examined, with  $\beta$  sheets likely to form only occasionally throughout the length of the sequence. The protein does not display any regions of marked hydrophobicity or hydrophilicity. The antigenic index (lane d) illustrates that *B. forsythus* hsp60 is predicted to be highly antigenic virtually throughout the length of the sequence. Some of these regions of predicted antigenicity correspond to regions of the amino acid

sequence likely to be expressed on the surface of the protein (lane b) which further implies their antigenic potential.

### ***3' DNA sequence of the hsp60 gene of P. intermedia***

A 1.6-kb PCR product containing the hsp60 gene of *P. intermedia* was generated using the same oligonucleotide primers and under the same PCR conditions as described previously when the hsp60 gene of *B. forsythus* was isolated. The PCR product was inserted into the plasmid vector pCR2.1-TOPO as described for the *B. forsythus* hsp60 gene in Chapter 3.

The DNA insert was sequenced using an automated sequencer and the commercially available primers M13 Forward and M13 Reverse. Sequencing data was analysed using the BasemagIR<sup>®</sup> software package which collected sequence data from the automated sequencer and provided a program for reading the bases. The DNA sequence was converted into FASTA format (compatible with BLAST searches) and on-line DNA sequence databases were searched for matches to identify the DNA sequence that the recombinant clone carried. DNA sequence databases were searched using the Institute for Genomic Research web-site at <http://www.tigr.org>.

The DNA sequence representing 653 nucleotides of the 3' terminal sequence of the hsp60 gene of *P. intermedia* is shown in Figure 4.6.

### ***Predicted amino acid sequence of the hsp60 protein of P. intermedia***

Translation of the sequenced portion of the 3' terminus of the groEL gene from *P. intermedia* revealed a single open reading frame of 217 amino acids. The predicted amino acid sequence is shown in Figure 4.6.

### ***Comparison of the predicted P. intermedia hsp60 amino acid sequence with those from other species***

The predicted amino acid sequence of the 3' terminus of the hsp60 gene from *P. intermedia*, representing 40% of the total hsp60 sequence, was aligned with the corresponding sequence of the hsp60 proteins of *B. forsythus*, *P. gingivalis*, *A. actinomycetemcomitans*, *E. coli* and human mitochondrial protein P1. This alignment is presented in Figure 4.7. For the portion of the gene shown, the *P. intermedia* sequence shares 71% amino acid homology with *B. forsythus* hsp60, 70% homology with *P. gingivalis*, 55% homology with *E. coli*, 54% homology with *A. actinomycetemcomitans* and 42% homology with the human homologue P1.

### ***Construction of a molecular phylogenetic tree***

A molecular phylogenetic tree was deduced from analysis of existing nucleotide sequences of hsp60 from a range of organisms along with that of *B. forsythus*. Phylogenetic analyses were performed using the PUZZLE (version 4.0.2) program in PHYLIP (version 3.5c). This program constructs trees from molecular sequence data by maximum likelihood, all sequence analysis used 1000 puzzling steps. The program tests whether the base composition of each sequence is identical to the average base composition of the whole alignment, and also computes the average distance between all pairs of sequences. The average distances can be viewed as a rough measure of the overall sequence divergence. Branches showing support values from 90 to 100% can be considered very strongly supported. The tree indicates a close evolutionary relationship between *B. forsythus* and *P. gingivalis*, confirming previous phylogenies.

## DISCUSSION

This chapter describes the DNA sequence of the hsp60 gene which codes for the groEL monomer of the hsp60 protein of *B. forsythus*. The nucleotide sequence representing the entire coding region of this gene was determined on both strands using overlapping oligonucleotide primers, and is described here for the first time. The DNA sequence of *B. forsythus* hsp60 was translated into a predicted protein sequence, and the derived amino acid sequence used to search for homologous proteins in on-line databanks using BLAST searches made available by the Institute of Genomic Research. As expected, virtually all members of the hsp60 family of chaperonins share an extremely high level of homology with *B. forsythus* hsp60.

The amino acid identity ranged from 81% with *P. gingivalis*, to 62% with *E. coli* and 50% with the gene product of human mitochondrial protein P1. The *B. forsythus* DNA sequence shares 73% identity with the nucleotide sequence of *P. gingivalis* hsp60, with some of the nucleotide differences occurring in the third or 'wobble' base of codons causing the higher level of amino acid homology. High levels of conserved amino acid residue changes have been described when the hsp60 peptide sequence of *Salmonella typhi* was compared to that of *E. coli* (Lindler and Hayes, 1994) and when the hsp60 sequences of various *Neisseria* species were analysed (Pannekoek *et al*, 1995). A broader study indicated that the minimum level of sequence identity between any two hsp60 sequences is 40%, beyond which a further 20% of the amino acid changes can be predicted to be conservative (Gupta, 1995).

Several well-conserved motifs and signature sequences have been identified



within the nucleotide and amino acid sequences of groEL. One motif consists of a Gly-Gly-Met repeat at the extreme terminal end of the sequence, with the number of repeats varying between species (Gupta, 1995; Pannekoek *et al*, 1995; Schmidt *et al*, 1992). This sequence is repeated twice in the *B. forsythus* hsp60 sequence, three times in the *P. gingivalis* sequence and four times in the *E. coli* sequence. Similar GGM motifs have also been observed in eukaryotic hsp60 homologues. Deletions at the C terminus of yeast hsp60 suggest that the presence of at least three glycine and methionine residues is essential for cell viability (Shu *et al*, 1991). A truncated version (minus GGM) of *E. coli* hsp60 has been constructed and hydrolyses ATP 1.5 times more slowly than wild type, and fails to suppress certain temperature-sensitive mutations (McLennan *et al*, 1991). Another motif, identified using the MOTIF programme of the Wisconsin Computer Group Package, is a conserved region of 12 amino acids located in the last third of the groEL sequence. The consensus pattern A-[A/S]-X-[E/Q]-E-X(4)-G-G-[G/A] has been found to exist in virtually all hsp60 sequences currently characterised, with the exception of mitochondrial *Plasmodium falciparum* groEL. The motif is located in the *B. forsythus* hsp60 sequence at residues 404-415 and is represented by the amino acid sequence AAIEEGTVPGGG. A signature that differentiates between sequences derived from Gram positive and Gram negative species has been located at residue 153 of *E. coli* hsp60 (which corresponds to residue 153 of *B. forsythus* hsp60 sequence). In Gram positive species, this residue is missing and a gap is created in the amino acid sequence as compared to Gram negative-derived sequences (Gupta, 1995).

As a result of their ubiquitous presence in prokaryotes and eukaryotic

organelles, universal conservation and considerable size, hsp60 sequences are ideal 'molecular clocks' that can be used to track evolutionary relationships between organisms, and to estimate at which period in the evolutionary time scale any sequence changes occurred. Due to their omnipresence and high level of sequence identity, it is apparent that all hsp60 sequences are derived from the sequence of an ancient common ancestor. Changes that have occurred in the sequence since then, for example the Gram positive 'missing' amino acid residue 153, can yield information on inter-bacterial relationships, allowing the construction of phylogenetic trees which plot their common ancestry. A phylogenetic tree constructed by Gupta (1995), rooted using a distant homologue of hsp60 from the archaebacterium *Sulfolobus shibatae*, arranges the hsp60 homologues in groups (Fig 4.8). These groups, and their order of branching in the evolutionary tree from the most ancient to the most recent group, provide a pattern that is consistent with observations made by other groups who have formed evolutionary trees using sequences from other genes in a similar manner. The hsp60 evolutionary tree indicates that the Gram positive bacteria, due to their deepest branching within the tree, represent the most ancient group studied. This ancient group includes examples of extremophiles and the mycobacteria. Midway through the evolutionary tree, a group diversifies that contains the spirochaetes and chlamydiae, and *P. gingivalis*. A molecular phylogenetic tree deduced from analysis of the nucleotide sequences of representatives from each different group and *B. forsythus* hsp60 sequence strongly supports the placement of *B. forsythus* in the same evolutionary group as *P. gingivalis* (Fig 4.9). More recently diversified than *B. forsythus* hsp60 is the group of mitochondrial hsp60 homologues.

Interestingly, the hsp60 phylogenetic tree supports the hypothesis described for the formation of eukaryotic organelles (mitochondria and chloroplasts), which are thought to have evolved following an endosymbiotic 'consumption' of particular groups of bacteria by an ancestral eukaryotic cell (Margulis, 1970). The high degree of amino acid identity conserved within a species may reflect evolutionary relatedness as Gupta implies, but it may also indicate that only minor sequence changes can be tolerated by a species. Perhaps each species' hsp60 is structurally adapted to best handle the proteins and polypeptides of that species, indicated by the relatively ineffective functional interchange of chaperonins between species.

The predicted  $\alpha$  helical structure of *B. forsythus* hsp60 (Fig 4.5) is in accordance with the predicted secondary structure of the hsp60 protein of mycobacteria (Young *et al*, 1987). Similar to the structural analysis of *B. forsythus* hsp60, the mycobacterial homologue displayed no strikingly hydrophobic regions, which contradicts data presented in a study of Neisserial hsp60 proteins (Pannekoek *et al*, 1985). This apparent difference may reflect the cellular localisation of hsp60 in different species. The lack of hydrophobic domains in the *B. forsythus* and mycobacterial sequences would make an association between these proteins and the lipid-rich cell wall of the species unlikely. However, the hydrophobic regions noted in the Neisserial sequence would permit such an interaction, and may indicate that Neisserial hsp60 could act to transport cellular proteins to the cell wall, or may chaperone proteins prior to secretion to maintain them in a conformation conducive to transport.

Notably hydrophilic regions of the *M. leprae* hsp65 predicted protein sequence have been shown to be major antibody-binding epitopes of the linear sequence

of the mycobacterial homologue to *B. forsythus* hsp60 (Anderson *et al*, 1988). Whilst hydrophilic domains do not feature in the *B. forsythus* hsp60 analysis, several of the antigenic regions identified match areas of the sequence which have been predicted to be expressed on the surface of the protein. Some of these areas of the protein, notably around residues 80, 360 and 390, are also amongst the best-conserved stretches of the *B. forsythus* hsp60 sequence. It is therefore possible that these are highly antigenic linear epitopes, or parts of discontinuous epitopes, that represent domains that may be important epitopes for inducing immune responses either as cross-reactive antibody binding sites or as epitopes recognised by T-lymphocytes.

Studies on the antigenic sites of hsp60 proteins of *Neisseria* species (Pannekoek *et al*, 1987) and mycobacteria (Young *et al*, 1987) support this postulation, with an area of the protein capable of binding a cross-reactive monoclonal antibody identified around residue 360. Interestingly, amino acid 361 was found to be a crucial determinant of the epitope with regards to *Neisseria* species binding the monoclonal antibody. *N. flavescens* was the only species in this study which did not bind the cross-reactive monoclonal antibody. Upon analysis, the *N. flavescens* sequence at the 360 region was found to differ in only one residue, at position 361. Thus it must be borne in mind that whilst well conserved domains may be identified and tagged as potentially cross-reactive epitopes, the alteration of a single amino acid within the conserved domain can deem a seemingly cross-reactive epitope to be species-specific. The high level of sequence identity that hsp60 homologues share has been well documented (Shinnick, 1991; Young *et al*, 1987) and *B. forsythus* shares this characteristically high level of homology, as seen in the alignment of hsp60

sequences from various species in Fig 4.4. Not only do these hsp60 homologues share sequence identity, but their genomic conservation reflects a conserved structure (a double ring 'doughnut' shape exhibiting 7-fold rotational symmetry) and function ('molecular chaperones') that all hsp60 chaperonins share.

Increasingly though, hsp60 proteins are being found to share a high level of sequence identity with a diverse array of apparently unrelated proteins. Dudani and Gupta (1989) initially observed this phenomenon after they had characterised the human hsp60 homologue, protein P1. Sequence alignments of the human hsp60 homologue revealed statistically significant alignments occurred with sequences coding for a number of seemingly unrelated proteins from pathogenic bacteria and viruses, including the pol polyprotein of immunodeficiency viruses, the  $\beta$  chain of cholera enterotoxin and the penicillin binding protein of *N. gonorrhoeae*. Jones *et al* (1993) sought to substantiate the implication that hsp60, due to its high level of sequence conservation, may be involved in autoimmune diseases. This they achieved by aligning the human hsp60 amino acid sequence, divided into 25-amino acid lengths that covered the entire gene, with the sequences of proteins which were 'known' autoantigens involved in a range of autoimmune diseases. Regions of the hsp60 sequence predicted to be potentially highly antigenic demonstrated sequence homology with 19 known autoantigens. Of these, the highest similarity scores were attributed to cytokeratin (associated with the autoimmune disease rheumatoid arthritis), neurofilament triplet M protein (multiple sclerosis), cardiac myosin heavy chain (coxsackie myocarditis) and cytochrome P450 (chronic active hepatitis). The human hsp60 protein also shared sequence

homology with proteins not currently identified as autoantigens, although the authors postulate that a role for these less-characterised proteins in autoimmune diseases may be proven in time.

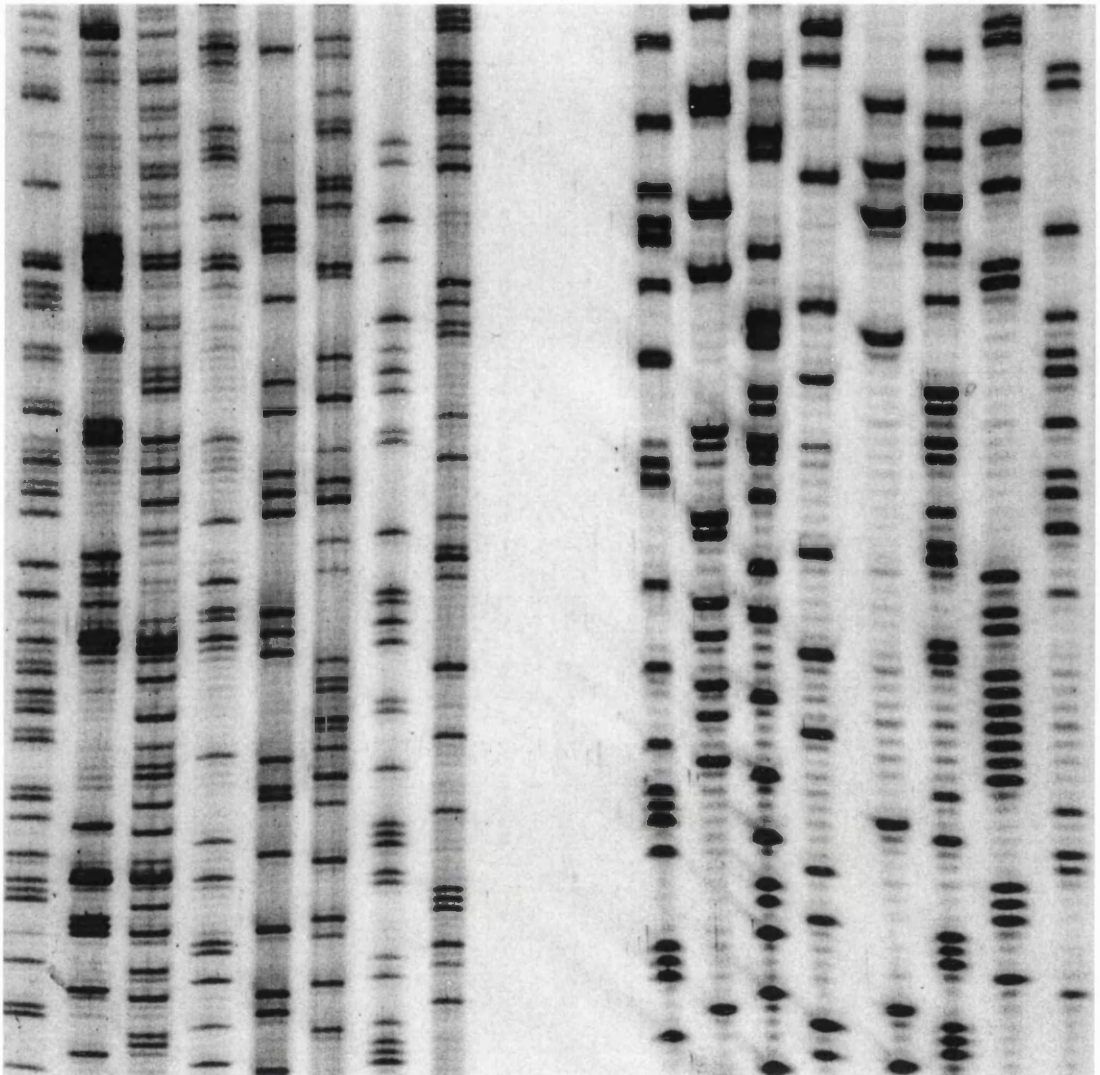
More recently, hsp60 shared sequences have been found in tissues and bacterial cells that have a degree of periodontal tissue specificity. The NH<sub>2</sub> terminus of a component of the surface-associated material (SAM) of *A. actinomycetemcomitans* shares >95% homology with the corresponding 38 residues from the NH<sub>2</sub> terminus of *E. coli* hsp60 (Kirby *et al*, 1995). As this protein has an apparent molecular weight of 62-kDa, and binds a monoclonal antibody that recognises *E. coli* and mycobacterial hsp60, it may be that this is less of a discovery of a new protein sharing hsp60 homology, but is more likely to be a further characterisation of a function that some hsp60 proteins may share, and also indicates the outer membrane localisation of hsp60 in *A. actinomycetemcomitans*.

Gemmell and Seymour (1995) refer to unpublished data which demonstrates that hsp60 proteins possess DNA sequence homologous to periodontal ligament fibroblast DNA. The periodontium-specific nature of these cells invites interesting speculation as to their involvement in autoimmune processes of periodontitis. Future work shall perhaps better characterise the protein from the ligament fibroblasts, and determine its antigenicity and potential for binding cross-reactive hsp60 antibodies.

In summary, this chapter presents the first report of the DNA sequence and predicted protein sequence of the hsp60 gene of *B. forsythus*, as well as a partial characterisation of the hsp60 DNA sequence of *P. intermedia*. Predicting some of the *B. forsythus* hsp60 secondary structures using the PEPLOT and

PEPTIDESTRUCTURE functions of the Genetics Computer Group Wisconsin programs, regions of the peptide that are likely to be immunogenic have been identified. By expressing the recombinant protein and assessing its reactivity with various antibody and serum preparations in Chapters 5 and 6, the assumption that *B. forsythus* hsp60 is an immunogenic protein bearing cross-reactive hsp60 antibody binding sites can be tested.

**FIGURE 4.1.** Autoradiograph of a typical sequencing gel obtained in determining the DNA sequence of the *B. forsythus* hsp60 gene.



**Figure 4.1.** Autoradiograph of a typical sequencing gel obtained in determining the DNA sequence of the *B. forsythus* hsp60 gene. Forward and reverse orientated custom-made primers were annealed to single-strand DNA generated from plasmid pBf1.6 and sequencing reactions then performed so that DNA was sequenced entirely in both directions. The samples shown on the gel are as follows:

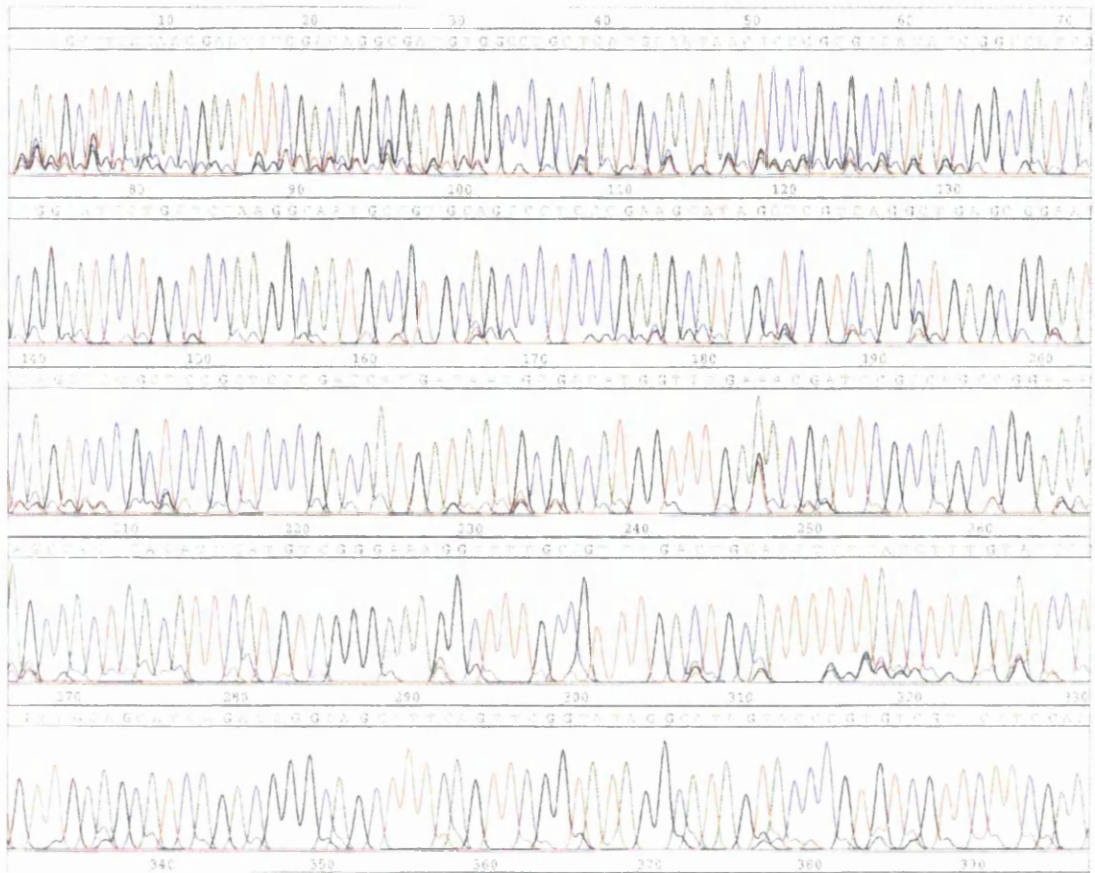
Forward primer (long run)- A track (lane 1), C track (lane 2), G track (lane 3), T track (lane 4); reverse primers (long run)- A track (lane 5), C track (lane 6), G track (lane 7), T track (lane 8); forward primer (short run)- A track (lane 9), C track (lane 10), G track (lane 11), T track (lane 12); reverse primer (short run)- A track (lane 13), C track (lane 14), G track (lane 15), T track (lane 16).

Thus, the first forward reaction (long run, lanes 1-4) begins reading at nucleotide position 78 of the sequence: ACGGTAAGCT....

The second forward reaction (short run, lanes 9-12) begins reading in this image at nucleotide position 367: GTATCGAAAG...



**FIGURE 4.2.** Example of sequence data derived from automatic DNA sequencing using LICOR 4200 system.



**Figure 4.2.** Example of sequence data obtained from automated DNA sequencing using the LICOR 4200 system. Commercially available and custom-made fluorescently labelled primers were annealed to denatured plasmid DNA, and cycle sequencing reactions performed. DNA was sequenced entirely in both directions. This image displays plus-strand DNA data.

**FIGURE 4.3. DNA sequence and predicted amino acid sequence of the *B. forsythus* hsp60 gene.**

ATGGCAAAGAAATCAAATTCGACATGAATGCCCCGCGACCTTTTGAAGAAAGGTGTGGAT	60
M A K E I K F D M N A R D L L K K G V D	20
GAATTGGCAAATGCCGTTAAGGTAACATTAGGTCCGAAAGGTCGCAATGTGATCCTCGAA	120
E L A N A V K V T L G P K G R N V I L E	40
AAGAAATTCGGTGCTCCGCAAATCACCAAAGACGGTGTAACGGTGGCGAAAGAAATAGAG	180
K K F G A P Q I T K D G V T V A K E I E	60
CTCGCTTGTCCGTACGAGAACATGGGGGCGCAGCTTGTGAAGGAAGTCGCTTCGAAGACG	240
L A C P Y E N M G A Q L V K E V A S K T	80
AACGACAAAGCCGGCGACGGTACGACAAC TGCTACCGTTT TGGCGCAAGCCATCAT TGGC	300
N D K A G D G T T T A T V L A Q A I I G	100
GTAGGATTGAAGAACGTAACGGCCGGGGCTAATCCAATGGACTTGAAACGCGGTATCGAC	360
V G L K N V T A G A N P M D L K R G I D	120
AAGGCCGTATCGAAAGTGGTAGAAAGCATCGCATCGCAGTCCGAAGCGGTAGGTACGAAC	420
K A V S K V V E S I A S Q S E A V G T N	140
ATGGACCGTATCGAGCATGTGGCTAAAATCTCGGCAAACGGCGATGAGGGTATCGGTAAG	480
M D R I E H V A K I S A N G D E G I G K	160
CTGATTGCCGAAGCGATGCAGAAGGTGAAGAAAGAAGGCGTCATCACGGTAGAAGAAGCC	540
L I A E A M Q K V K K E G V I T V E E A	180
AAGGGAACGGAAACGACCGTGGAGGTTGTGGAAGGTATGCAGTTCGATCGCGTTTATATC	600
K G T E T T V E V V E G M Q F D R G Y I	200
TCCGCTTATTTTCGTGACCGATACGGAAAAGATGGAAACACAGTTCGAAAATCCGTACATT	660
S A Y F V T D T E K M E T Q F E N P Y I	220
CTGATCTACGATAAAAAGATTTTCAGTGCTGAAAGACCTGCTTCCCATCCTGGAGCAAATG	720
L I Y D K K I S V L K D L L P I L E Q M	240
GTTCAGTCGGGTCGTGCTTTGCTGATCATTTGCCGAAGACATCGACAGCGAAGCGTTGGCT	780
V Q S G R A L L I I A E D I D S E A L A	260
ACGCTTGTGGTGAACCGTCTGCGCGGTGGTTTGAAGTGTGCGCCGTGAAGGCTCCGGGC	840
T L V V N R L R G G L K V C A V K A P G	280
TTCGGCGACCGTCGTAAGGCTATGCTCGAAGATATTGCCATCTTGACAGGTGGAACGGTG	900
F G D R R K A M L E D I A I L T G G T V	300
ATTACCGAAGAAAAAGGCATGAAACTGGAAGATGCCAAGATGGATATGCTCGGGTCGGCC	960
I T E E K G M K L E D A K M D M L G S A	320
GACAAGGTCACGGTGAACAAAGACAATACGACGATCGTGAAAGGGAACGGCGACAAGGCT	1020
D K V T V N K D N T T I V K G N G D K A	340
GCTATTGAGTCTCGTATCGGCCAGATCAAGGCACAGATCGAAACAACGACATCGGACTAC	1080

A I E S R I G Q I K A Q I E T T T S D Y	360
GACAAGGAGAAGTTGCAGGAACGTTTGGCGAAGTTGGCCGGCGGAGTCGCCGTGCTCTAC	1140
D K E K L Q E R L A K L A G G V A V L Y	380
GTCGGCGCTCCTTCGGAAGTGGAAATGAAAGAGAAGAAAGACCGTGTAGACGACGCCTTA	1200
V G A P S E V E M K E K K D R V D D A L	400
CATGCTACCCGCGCCGCGATCGAAGAAGGTACGGTGGCCGGCGGTGGAGTCGCTTATCTT	1260
H A T R A A I E E G T V P G G G V A Y L	420
CGCGCGATCCCGGCACTCGAAGGCCTCAAGGGGGAGAACGAAGACGAAACGACGGGTATC	1320
R A I P A L E G L K G E N E D E T T G I	440
GAGATTGTGAAACGCGCCATCGAAGAGCCGTTGCGTCAGATCGTGAACAACGCCGGTAAG	1380
E I V K R A I E E P L R Q I V N N A G K	460
GAAGGTGCGGTAGTGGTGCAGAAAGTGAAGAAGGCACAGGTGCTTTCGGATACAACGCT	1440
E G A V V V Q K V K E G T G A F G Y N A	480
CGTACCGATGTGTACGAAGACCTCTCTGAGGCCGGAGTCGTTGACCCTGCCAAAGTGACA	1500
R T D V Y E D L S E A G V V D P A K V T	500
CGTATCGCCCTTGAGAATGCCGCATCGATTGCCGGCATGTTTCCTGACCACCGAATGCGTC	1560
R I A L E N A A S I A G M F L T T E C V	520
GTAGCCGATAAGAAGGAAGAGGCACCGGCCCCCCCCGATGAACCCGGGCATGGGTGGCATG	1620
V A D K K E E A P A P P M N P G M G G M	540
GGCGGCATGATGTAA	1635
G G M M *	544

**FIGURE 4.3.** DNA sequence and predicted amino acid sequence of the coding region of the *B. forsythus* hsp60 gene. The predicted amino acid sequence is shown below the nucleotide sequence. The amino acid sequence constitutes a single open reading frame of 544 amino acids.

**FIGURE 4.4. Alignment of the *B. forsythus* and *P. gingivalis* hsp60 DNA sequences.**

<i>B. f.</i> hsp60	ATGGCAAAAGAAATCAAATTCGACATGAATGCCCGCG	37
<i>P. g.</i> hsp60	ATGGCAAAAGAAATCAAATTCGATATGGAATCTCGCG	37
	ACCTTTTGAAGAAAGGTGTGGATGAATTGGCAAATGCCGTTAAGGTAACA	87
	ACCTTCTGAAGAAGGGCGTAGATGCACTGCCAAATGCCGTTAAAGTTACC	87
	TTAGGTCCGAAAGGTGCGCAATGTGATCCTCGAAAAGAAATTCGGTGCTCC	137
	CTCGGGCCGAAAAGTTTCGTAACGTTATCCTTAGCAAGACGTACGGAGCTCC	137
	GCAAATCACCAAAGACGGTGTAACGGTGGCGAAAAGAAATAGAGCTCGCTT	187
	GCACATTACCAAGGACGGTGTGAGCGTAGCGAAAGAGATAGAATTGGAGT	187
	GTCCGTACGAGAACATGGGGGCGCAGCTTGTGAAGGAAGTCGCTTCGAAG	237
	GCCCGTTTCGAGAACATGGGTGCCCAGTTGGTGAAAGAAGTAGCCTCCAAG	237
	ACGAACGACAAAGCCGGGCGACGGTACGACAACTGCTACCGTTTTGGCGCA	287
	ACCAATGACGATGCCGGTGACGGTACGACTACGGCTACGATCCTCGCCCA	287
	AGCCATCATTGGCGTAGGATTGAAGAACGTAACGGCCGGGGCTAATCCAA	337
	GAGCATTATCGGCGTGGGTCTGAAGAACGTTACGGCAGGAGCCAATCCGA	337
	TGGACTTGAAACGTGGTATCGACAAGGCCGTATCGAAAGTGGTAGAAAGC	387
	TGGATTTGAACCGTGGTATCGACAAGTCTGTAAAGTCTGTGGTAACTCAC	387
	ATCGCATCGCAGTCCGAAGCGGTAGGTACGAACATGGACCGTATCGAGCA	437
	ATCGCAGGTATGGCTAAGGAAGTGGGCGCCGACTTCCAGAAGATCGAGCA	437
	TGTGGCTAAAATCTCGGCAAACGGCGATGAGGGTATCGGTAAGCTGATTG	487
	CGTAGCCAAGATCTCTGCCAACGGAGACGAAAACATCGGTAGCCTCATTG	487
	CCGAAGCGATGCAGAAGGTGAAGAAAGAAGGGGTCATCACGGTAGAAGAA	537
	CCGAAGCCATGCGCAAGGTAAAGAAAGAAGGCCTTATCACGGTAGAAGAA	537
	GCCAAGGGAACGGAAACGACCGTGGAGGTTGTGGAAGGTATGCAGTTCGA	587
	GCCAAGGGAACCGACACTACGGTAGAAGTGGTGGAAAGGTATGCAGTTCGA	587
	TCGCGGTTATATCTCCGCTTATTTTCGTGACCGATACGGAAAAAGATGGAAA	637
	CCGCGGCTACATCTCTCCCTACTTCGTGACGAATACGGATAAGATGGAGG	637
	CACAGTTCGAAAATCCGTACATTCTGATCTACGATAAAAAGATTTTCAGTG	687
	TCCAAATGGAAAATCCTTTCATCCTCATCTACGACAAGAAAATATCCGTG	687

CTGAAAGACCTGCTTCCCATCCTGGAGCAAATGGTTTCAGTCGGGTCGTGC	737
CTGAAAGAGATGCTCCCGATCCTCGAACAAACGGTTCAGACGGGCAAGCC	737
TTTGCTGATCATTGCCGAAGACATCGACAGCGAAGCGTTGGCTACGCTTG	787
CCTCCTCATCATTGCAGAAGACAACGACAGCGAAGCACTCCCTACCTTGG	787
TGGTGAACCGTCTGCGCGGTGGTTTGAAAGTGTGCGCCGTGAAGGCTCCG	837
TTGTAAACCGTCTGCGCGGCAGCCTCAAGATCTGTGCAGTGAAGGCTCCC	837
GGCTTCGGCGACCGTCGTAAGGCTATGCTCGAAGATATTGCCATCTTGAC	887
GGATTTCGGCGATCGTCGCAAGGCTATGCTCGAAGACATTGCTATCCTGAC	887
AGGTGGAACGGTGATTACCGAAGAAAAAGGCATGAACTGGAAGATGCCA	937
GGGCGGAGCGGTTATCAGCGAAGAGACCGGTCTGAACCTCGAAAATACTA	937
AGATGGATATGCTCGGGTCGGCCGACAAGGTCACGGTGAACAAAGACAAT	987
CGATGGATATGCTCGGTACAGCTGAGAAAGTAAGGGTTGATAAGACAAT	987
ACGACGATCGTGAAAGGGAACGGCGACAAGGCTGCTATTGAGTCTCGTAT	1037
ACTACTATCGTTAACGGAGCCGGAAACAAAGAAGGCATCGCTTCACGTAT	1037
CGGCCAGATCAAGGCACAGATCGAAACAACGACATCGGACTACGACAAGG	1087
CACGCAGATCAAAGCTCAGATCGAGAATACGACCAGCGACTACGACCGCG	1087
AGAAGTTGCAGGAACGTTTGGCGAAGTTGGCCGGCGGAGTCGCCGTGCTC	1137
AAAAGCTGCAAGAACGTTTGGCCAAGCTCGCCGGCGGTGTAGCTGTTCTT	1137
TACGTCGGCGCTCCTTCGGAAGTGGAATGAAAGAGAAGAAAGACCGTGT	1187
TACGTGGGTGCTGCCAGCGAAGTGGAATGAAGGAAAAGAAGGATCGCGT	1187
AGACGACGCCTTACATGCTACCCGCGCCGCGATCGAAGAAGGTACGGTGC	1237
AGAAGATCCCTTGAGTCCAACGCGTCCTCCAATCGAAGAGGGTACAGTAC	1237
CCGGCGGTGGAGTCGCTTATCTTCGCGCGATCCCGGCACTCGAAGGCCTC	1287
CCGGTGGCGGTACGACATACATTCTGTGCCATAGCTGCTTTGGAAGGTCTC	1287
AAGGGAGAGAACGAAGACGAAACGACGGGTATCGAGATTGTGAAACGCGC	1337
AAGGGTGAGAACGAAGACGAAACCACAGGTATCGAGATCGTGAAACGCGC	1337
CATCGAAGAGCCGTTGCGTCAGATCGTGAACAACGCCGGTAAGGAAGGTG	1387
CATCGAGGAGCCGCTTCGTCAGATCGTAGCGAACGCCGGTAAAGAGGGTG	1387

CCGTAGTGGTG CAGAAAGTGAAAGAAGGCACAGGTGCTTTCCGGATACAAC	1437
CCGTTATGGTACAGAAAGGTGAAAGAAGGCAAGGACGACTTCGGCTACAAT	1437
GCTCGTACCGATGTGTACGAAGACCTCTCTGAGGCCGGAGTCGTTGACCC	1487
GCCCGTACGGATGTTTTCGAAAACTCTACACCACCGGTGTTATCGACCC	1487
TGCCAAAGTGACACGTATCGCCCTTGAGAATGCCGCATCGATTGCCGGCA	1537
AGCCAAAGTAACACGTGTTCATTGGAAAATGCAGCGTCTATCGCAGGTA	1537
TGTTTCCTGACCACCGAATGCGTCGTAGCCGATAAGGAGGAAGAGGCACCG	1587
TGTTTCCTGACTACGGAGTGCCTTATCGCTGACAAGAAAGAAGATAATCCT	1587
GCCCCCCCCG---ATGAACCCGGGCATGGGTGGCATGGGCGGCATGATGTA	1634
GCCGCACCGGCCATGCCCGAGGTATGGGAGGAATGGGCGGCATGATGTA	1637
A-----	1635
AACATCGATGCGAACAGGATGCGTTTTTTGATTCTTTTCGAAAAAATATC	1687
-----	
CTGTAGGACATTTCATATCACATTACAAGCCGAAGGCTGTGCAGAGGGTTC	1937
-----	
TCCCTCACTGCACAGGCTTCATTTTGGACTT	1968

**Figure 4.4.** Alignment of the *B. forsythus* (upper sequence) and *P. gingivalis* (lower sequence) hsp60 DNA sequences. Identical residues are indicated with a vertical bar, and dashes denote spaces introduced to maximise homology between the two species.

**FIGURE 4.5. Alignment of the *B. forsythus* hsp60 amino acid sequence with hsp60 sequences from other species.**

Bf	-----MAKEIKFDMNARDLLKKGVDELA	23
Aa	-----MAAKDVKFGNDARVKMLNGVNILA	24
Ec	-----MAAKDVKFGNDARVKMLRGVNVLA	24
Pg	-----MAKEIKFDMESRDLLKKGVDA LP	23
P1	--MLRLPTVFRQMRPVSRLAPHLTRAYAKDVKFGADARALMLQGVDLLA	48
Con	-----AK--KF----R-----GV--L-	
Bf	NAVKVTLGPKGRNVILEKKFGAPQITKDGVTVAKEIELACPYENMGAQLV	73
Aa	DAVKVTLGPKGRNVLDKSF GAPTITKDGVS VAREIELEDKFENMGAQMV	74
Ec	DAVKVTLGPKGRNVLDKSF GAPTITKDGVS VAREIELEDKFENMGAQMV	74
Pg	NAVKVTLGPKVRNVILSKTYGAPHITKDGVSVAKEIELECPFENMGAQLV	73
P1	DAVAVTMGPKGRVTVII EQSWGSPKVTKDGVTVAKSIDLKDKYKNIGAKLV	98
Con	-AV-VT-GPK-R-V-----G-P--TKDGV-VA--I-L-----N-GA--V	
Bf	KEVASKTNDKAGDGT TTTATVLAQAIIGVGLKNVTAGANPMDLKR GIDKAV	123
Aa	KEVASKANDAAGDGT TTTATVLAQAI VNEGLKAVAAGMNPMDLKR GIDKAV	124
Ec	KEVASKANDAAGDGT TTTATVLAQAI ITEGLKAVAAGMNPMDLKR GIDKAV	124
Pg	KEVASKTND DAGDGT TTTATILAQSIIGVGLKNVTAGANPMDLNR GIDKSV	123
P1	QDVANNTNEEAGDGT TTTATVLARSIAKEGF EKISKGANPVEIRRGV MLAV	148
Con	--VA---N--AGDGT TTTAT-LA--I---G-----G-NP----RG----V	
Bf	SKVVESIASQSEAVGTNMDRIEHVAKISANGDEGIGKLIAEAMQKV KKEG	173
Aa	NSVVAELKNLSKPC-ETSKEIEQVGTISANSDSIVGQLIAQAMEKV GKEG	173
Ec	TAAVEELKALSVPC-SDSKAIAQVGTISANSDET V GKLI AEAMDKV GKEG	173
Pg	KSVVTHIAGMAKEVGAD FQKIEHVAKISANGDENIGSLIAEAMRKV KKEG	173
P1	DAVIAELKKQSKPV-TTPEEIAQVATISANGDKEIGNIISDAMKKVGR KG	197
Con	-----I--V--ISAN-D---G--I--AM-KV---G	
Bf	VITVEEAKGTETTVEVVEGMQFDRGYISAYFVTDTEKMETQFENPY ILIY	223
Aa	VITVEDGTGLEDEL DVVEGMQFDRGYLSPYFINKPETATVELDNPFILLV	223
Ec	VITVEDGTGLQDEL DVVEGMQFDRGYLSPYFINKPETGAVELES PFILLA	223
Pg	VITVEEAKGTDTTVEVVEGMQFDRGYISPYFVTNTDKMEVQ MENPFIL IY	223
P1	VITVKDGKTLNDELEI IEGMKFDRGYISPYFINTSKGQKCE FQDAYV LLS	247
Con	VITV-----EGM-FDRGY-S-YF-----L--	
Bf	DKKISVLKDLLPILEQMVQSGRALLIIAEDIDSEALATLVVNRLRGGLKV	273
Aa	DKKISNIRELLPVLEGVAKAGKPLLI IAEDVEGEALATLVVNTMRGIVKV	273
Ec	DKKISNIREMLPVLEAVAKAGKPLLI IAEDVEGEALATAVVNTIRGIVKV	273
Pg	DKKISVLKEMLPILEQTVQTGKPLLI IAEDNDSEALPTLVVNRLRGS LKI	273
P1	EKKISSIQSIVPALEIANAHRKPLVIIAEDVDGEALSTLVNRLKVGLQV	297
Con	-KKIS-----P-LE-----L-IIAED---EAL-T-V-N-----	
Bf	CAVKAPGFGDRRKAMLEDIAILTGGTVITEE-KGMKLEDAKMDMLGSADK	322
Aa	AAVKAPGFGDRRKAMLQDIAILTAGTVISEE-IGMELEKATLEDLGQAKR	322
Ec	AAVKAPGFGDRRKAMLQDIAILTGGTVISEE-IGMELEKATLEDLGQAKR	322
Pg	CAVKAPGFGDRRKAMLEDIAILTGGAVISEE-TGLNLENTTMDMLGTAEK	322
P1	VAVKAPGFGDNRKNQLKDMAIATGGAVFGEEGLTLNLEDVQPHDLGKVGE	347
Con	-AVKAPGFGD-RK--L-D-A--T-G-V--EE-----LE-----LG----	

Bf	VTVNKDNTTIVKGNKDAAIESRIGQIKAQIETTTSDYDKEKLQERLAKL	372
Aa	IVINKDNTTIIIDGIGDEAQIQGRVAQIRQQIEESTSDYDKEKLQERVAKL	372
Ec	VVINKDTTTIIIDVGEEAAIQGRVAQIRQQIEEATSDYDREKLQERVAKL	372
Pg	VRVDKDNTTIVNGAGNKEGIASRITQIKAQIENTTSDYDREKLQERLAKL	372
P1	VIVTKDDAMLLKGKGDKAQIEKRIQEIIIEQLDVTTSEYEKEKLNERLAKL	397
Con	----KD-----G-G----I--R---I--Q----TS-Y--EKL-ER-AKL	
Bf	AGGVAVLYVGAPSEVEMKEKKDRVDDALHATRAAIEEGTVPGGGVAYLRA	422
Aa	AGGVAVIKVGAATEVEMKEKKARVEDALHATRAAVEEGIVAGGGVALIRA	422
Ec	AGGVAVIKVGAATEVEMKEKKARVEDALHATRAAVEEGVVAGGGVALIRV	422
Pg	AGGVAVLYVGAASEVEMKEKKDRVEDPLSPTRPPIIEGTVPGGGTTYIRA	422
P1	SDGVAVLKVGGTSDVEVNEKKDRVTDALNATRAAVEEGIVLGGGCALLRC	447
Con	--GVAV--VG-----VE--EKK-RV-D-L--TR---EEG-V-GGG-----R-	
Bf	IPALEGLKGENEDETTGIEIVKRAIEEPLRQIVNAGKEGAVVVQKVKEG	472
Aa	AGRVVGLQGENEEQNVGIKVALRAMEAPLRQIVANAGEEASVIASAVKNG	472
Ec	ASKLADLRGQNEQNVGIKVALRAMEAPLRQIVLNCGEEPSVVANTVKG	472
Pg	IAALEGLKGENEDETTGIEIVKRAIEEPLRQIVANAGKEGAVMVQKVKEG	472
P1	IPALDSLTPANEDQKIGIEIIKRTLKIPAMTIKNAGVEGSLIVEKIMQS	497
Con	-----L---NE----GI-----R-----P---I--N-G-E-----	
Bf	TGAFGYNARTDVYEDLSEAGVVDPAKVTRIALENAASIAGMFLTTECVVA	522
Aa	EGNFGYNAGTEQYGDMIAMGILDPTKVTRISALQFAASVAGLMITTECMVT	522
Ec	DGNYGYNAATEEYGNMIDMGILDPTKVTRISALQYAASVAGLMITTECMVT	522
Pg	KDDFGYNARTDVFNLYTTGVIDPAKVTRVALENAASIAGMFLTTECVIA	522
P1	SSEVGYDAMAGDFVNMVEKGIIDPTKVVRTALLDAAGVASLLTTAEVVVT	547
Con	----GY-A-----G--DP-KV-R-AL--AA--A----T-E----	
Bf	DKKEEAPAPPMNP-GMGMGGM	544
Aa	ELPKDDKADLGAA-GMGMGGMGGM	547
Ec	DLPKNDAAADLGAAGGMGMGMGM	548
Pg	DKKEDNPAAPAMPGGMGGMGM	545
P1	EIPKEEKDP--GMGAMGMGMGMGMGMF	573
Con	-----MGMGMG-----	

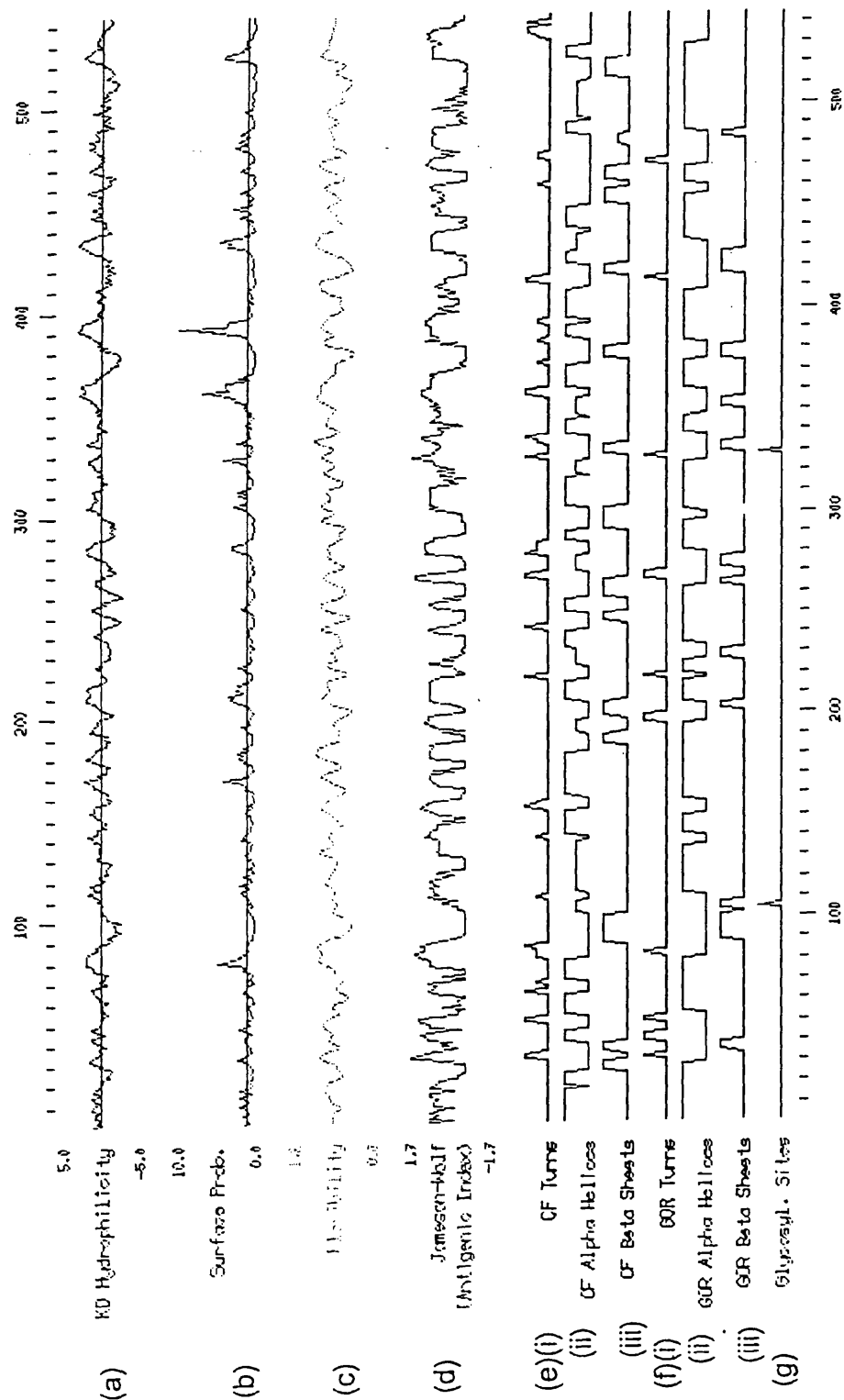
**FIGURE 4.5.** Alignment of the amino acid sequences of the hsp60 proteins of *Bacteroides forsythus* (Bf), *Actinobacillus actinomycetemcomitans* (Aa), *Escherichia coli* (Ec), *Porphyromonas gingivalis* (Pg) and human mitochondrial protein P1 (P1). Dashes denote spaces introduced to maximise homology between the sequences. The consensus sequence (Con) denotes residues that are identical in all five proteins.



**Figure 4.6.** Predicted secondary structure of *B. forsythus* hsp60. (a) Hydropathic plots determined by the method of Kyte and Doolittle (1982). Points above the horizontal line represent regions of above average hydrophobicity. (b) Plots of surface probability determined by the method of Emami *et al* (1985). (c) Plots of chain flexibility determined by the Karplus-Schulz method. (d) Plots of antigenic index determined by the method of Jameson and Wolf (1988). Antigenic index is a measure of the probability that a region is antigenic and is calculated by the summation of several weighted measures of secondary structure such as hydrophilicity, surface probability and chain flexibility. (e) Secondary structure analysis by the method of Chou and Fasman (1978) (CF) showing predicted turns (i), alpha helices (ii), and beta sheets (iii). (f) Secondary structure analysis by the method of Garnier *et al* (1978) (GOR) showing predicted turns (i), alpha helices (ii) and beta sheets (iii). (g) Potential N glycosylation sites. Height of the vertical bar represents the probability of glycosylation.

All analyses were carried out using PEPTIDESTRUCTURE and PLOTSTRUCTURE from the University of Wisconsin Genetics Computer Group programmes (Devereux *et al*, 1984).

FIGURE 4.6. Predicted secondary structures of the *B. forsythus* hsp60 amino acid sequence.



**FIGURE 4.7. DNA sequence and predicted amino acid sequence of the C terminal fragment of the *P. intermedia* hsp60 gene.**

GGTGTGTGTTATCXGCGAAGATAAGGGTTTGACACTCGACAAGGCAACACTC	50
G V V I X E D K G L T L D K A T L	
GAAATGTTGGGTTTCAGCTAAAAAGGTTACCGTTTCTAAGGATTTCCACCAAC	100
E M L G S A K K V T V S K D F T N	
ATCGTAGACGGTGCAGGCTCTAAGGAAAGCATTGCAGAGCGTGTGAATGCT	150
I V D G A G S K E S I A E R V N A	
ATCAAGAGCGAAATTGCGAACACAAAGAGCCAGTACGATAAAGAGAAACTA	200
I K S E I A N T K S Q Y D K E K L	
CAAGAGCGTTTGGCAAAACTTGCTGGTGGCGTTGCAGTGCTTTACGTGGGT	250
Q E R L A K L A G G V A V L Y V G	
GCCAACTCTGAAGTAGAGATGAAAGAGAAGAAAGACCGTGTAGACGATGCA	300
A N S E V E M K E K K D R V D D A	
CTCTGTGCGACTCGTGCAGCAACCGAAGAAGGTGTTGTTGTAGGTGGCGGT	350
L C A T R A A T E E G V V V G G G	
ACTACTTATATCCGTGCTCAAGAGGTTTTTGAAAGTCCTTACAGGCGAAAAT	400
T T Y I R A Q E V L K V L T G E N	
CCTGACGAACAGACAGGTATCAATATTGTTTGCCGTGCCATTGAAGAACCA	450
P D E Q T G I N I V C R A I E E P	
CTTCGCCAGATTGTTTATAACGCTGGTGGCGAGGGTGCAGTAGTTGTCAAT	500
L R Q I V Y N A G G E G A V V V N	
AAGGTACGCGAAGGCAAGGGCGACTTCGGTTACTACGCGCGTCGCGACCAG	550
K V R E G K G D F G Y Y A R R D Q	
TACGAAGACTTGCGTGCAGCAGGTGTTATCGACCCCGCAAAGGTTTCTCGT	600
Y E D L R A A G V I D P A K V S R	
GTAGCTTTGGAAAATGCAGCTTCAATCGCTGGTATGTTC	638
V A L E N A A S I A G M F	

**Figure 4.7.** DNA sequence and predicted amino acid alignment of the C terminal portion of the *P. intermedia* hsp60 gene. The predicted amino acid sequence is shown below the nucleotide sequence.

**Figure 4.8.** Alignment of *P. intermedia* hsp60 amino acid sequence (Pi) with amino acid sequences of the hsp60 proteins of *A. actinomycetemcomitans* (Aa), *E. coli* (Ec), *B. forsythus* (Bf), *P. gingivalis* (Pg) and human mitochondrial protein (P1). Dashes denote spaces introduced to maximise homology between the sequences. The consensus sequence (Con) denotes residues that are identical in at least four of the six proteins.

**FIGURE 4.8. Alignment of the *P. intermedia* hsp60 amino acid sequence with hsp60 sequences from other species.**

Aa	AAVKAPGFGDRRKAMLQDIAILTAGTVISEE-IGMELEKATLEDLGQAKR
Ec	AAVKAPGFGDRRKAMLQDIATLTGGTVISEE-IGMELEKATLEDLGQAKR
Bf	CAVKAPGFGDRRKAMLEDIAILTTGGTVITEE-KGMKLEDAKMDMLGSADK
Pg	CAVKAPGFGDRRKAMLEDIAILTTGGAVISEE-TGLNLENTTMDMLGTAEK
Pi	-----GVVIXED-KGLTLDKATLEMLGSAKK
P1	VAVKAPGFGDNRKNQLKDMAIATGGAVFGEEGLTLNLEDVQPHDLGKVGE
Con	-----G-VI-EE-G---LE-AT---LG-A--
Aa	IVINKDNTTIIIDGIGDEAQIQGRVAQIRQQIEESTSDYDKEKLQERVAKL
Ec	VVINKDTTIIIDGVGEEAAIQGRVAQIRQQIEEATSDYDREKLQERVAKL
Bf	VTVNKDNTTIVKGNKDAAIESRIGQIKAQIETTTSDYDKEKLQERLAKL
Pg	VRVDKDNTTIVNGAGNKEGASRITQIKAQIENTTSDYDREKLQERLAKL
Pi	VTVSKDFTNIVDGAGSKESIAERVNAIKSEIANTKSQYDKEKLQERLAKL
P1	VIVTKDDAMLLKGKGDKAQIEKRIQEIIIEQLDVTTSYEKEKLNERLAKL
Con	V-V-KD-TTI--G-G-KA-I--R--QI--QIE-TTSDYDKEKLQERLAKL
Aa	AGGVAVIKVGAATEVEMKEKKARVEDALHATRAAVEEGIVAGGGVALIRA
Ec	AGGVAVIKVGAATEVEMKEKKARVEDALHATRAAVEEGVAVAGGGVALIRV
Bf	AGGVAVLYVGAPSEVEMKEKKDRVDDALHATRAAIEEGTVPGGGVAYLRA
Pg	AGGVAVLYVGAASEVEMKEKKDRVEDPLSPTRPPIIEGTVPGGGGTTYIRA
Pi	AGGVAVLYVGANSEVEMKEKKDRVDDALCATRAATEEGVVVGGGGTTYIRA
P1	SDGVAVLKVGGTSDVEVNEKKDRVTDALNATRAAVEEGIVLGGGCALLRC
Con	AGGVAVL-VGA-SEVEMKEKKDRV-DAL-ATRAA-EEG-V-GGG-A-IRA
Aa	AGRVVGLQGENEEQNVGIKLALRAMEAPLRQIVANAGEEASVIASAVKNG
Ec	ASKLADLRGQNEQNVGIKVALRAMEAPLRQIVLNCGEPSVNVANTVKGG
Bf	IPALEGLKGENEDETGTGIEIVKRAIEEPLRQIVNAGKEGAVVQKVKEG
Pg	IAALEGLKGENEDETGTGIEIVKRAIEEPLRQIVANAGKEGAVMVQKVKEG
Pi	QEVCLKVLTGENPDEQGTGINIVCRAIEEPLRQIVYNAGGEGAVVNVKREG
P1	IPALDSLTPANEDQKIGIEIIKRTLKIPAMTIAKNAGVEGSLIVEKIMQS
Con	---L--L-GENED---GI-I--RA-E-PLRQIV-NAG-EG-V-V-KVK-G
Aa	EGNFGYNAGTEQYGDMIAMGILDPTKVTRSALQFAASVAGLMITTECMVT
Ec	DGNYGYNAATEEYGNMIDMGILDPTKVTRSALQYAASVAGLMITTECMVT
Bf	TGAFGYNARTDVYEDLSEAGVVDPAKVTRIALENAASIAGMFLTTECVVA
Pg	KDDFGYNARTDVFENLYTTGVIDPAKVTRVALENAASIAGMFLTTECVIA
Pi	KGDFGYYYARRDQYEDLRAAGVIDPAKVSRALENAASIAGMF-----
P1	SSEVGYDAMAGDFVNMVEKGIIDPTKVVRTALLDAAGVASLLTTAEVVVT
Con	-G-FGYNA-T--Y-----G--DP-KVTR-AL--AAS-AG---TTEC-V-
Aa	ELPKDDKADLGAA-GMGGMGGMGMM
Ec	DLPKNDAADLGAAGMGGMGGMGMM
Bf	DKEEEAPAPPMNP-GMGGMGGM
Pg	DKKEDNPAAPAMPGMGGMGGM
Pi	-----
P1	EIPKEEKDP--GMGAMGGMGGMGGMGGMF
Con	-----A-----GMGGMGGM-----

FIGURE 4.9. Evolutionary tree based on hsp60 sequences

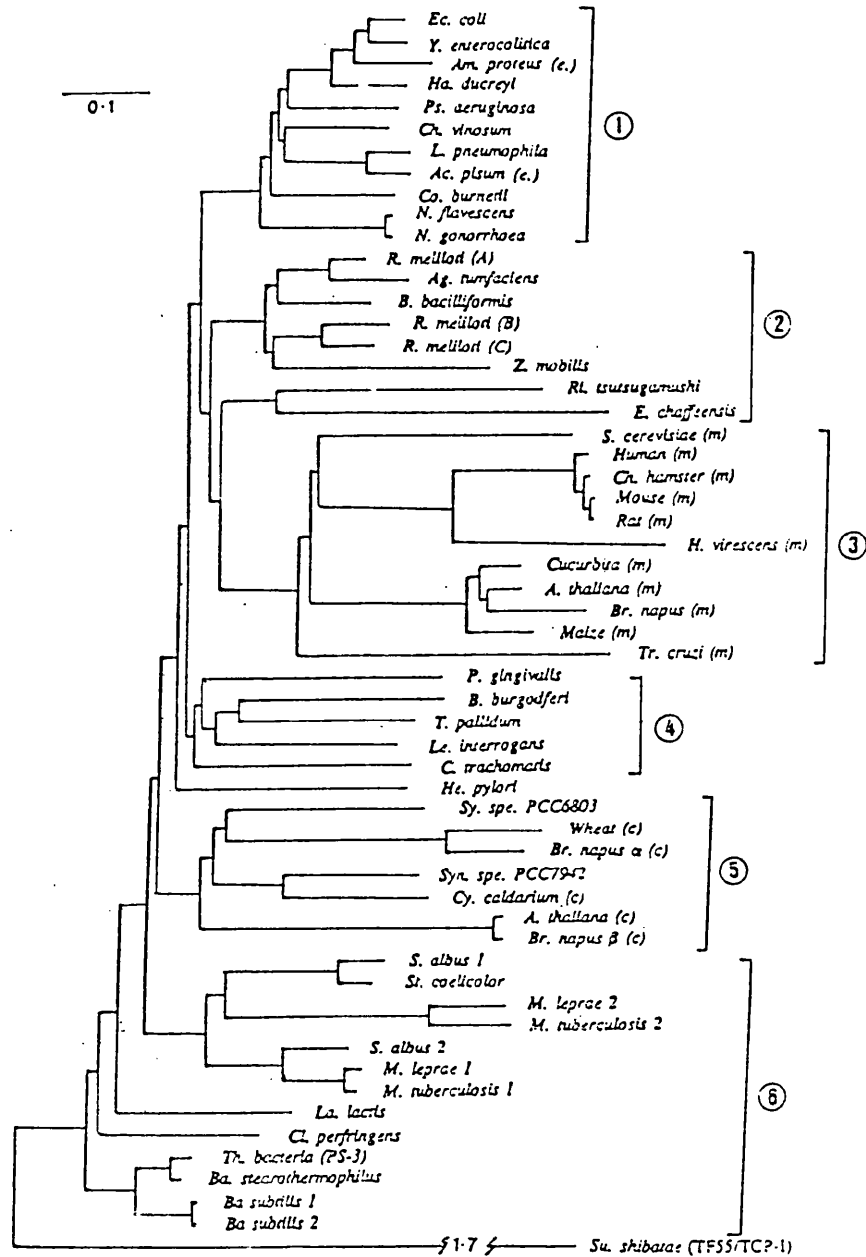
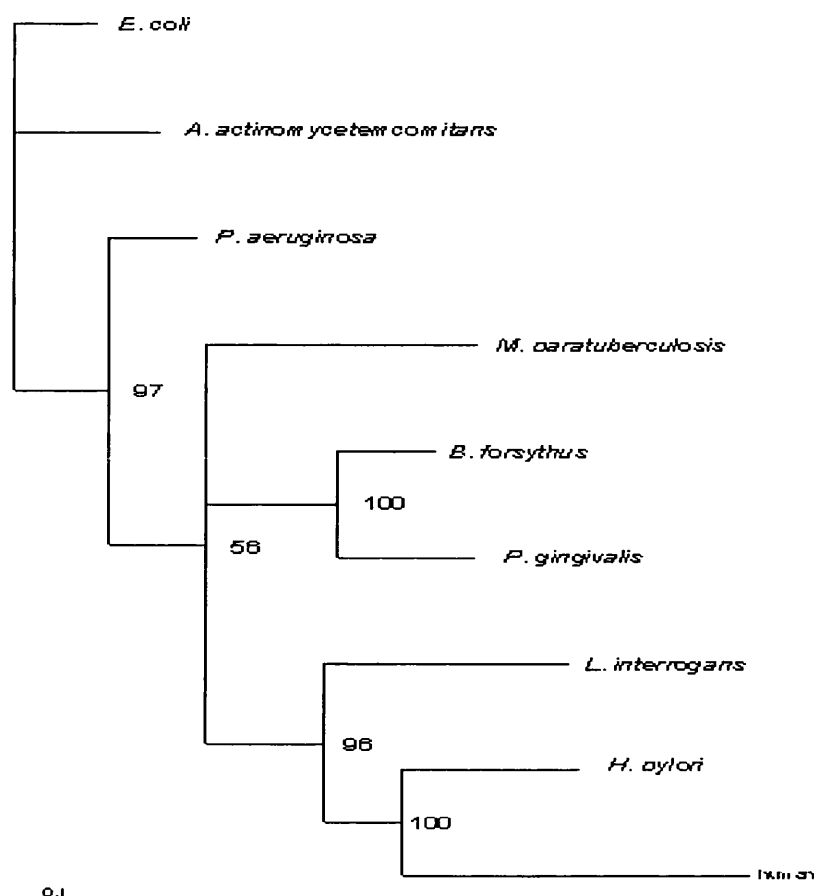


Figure 4.9. Evolutionary tree based on hsp60 sequences (Gupta, 1995). The tree was rooted using the Tcp-1 protein (hsp60 homologue) from *Sulfolobus shibatae*. The numbers 1 to 6 identify different groups of sequences corresponding to  $\beta$ - and  $\gamma$ -purple bacteria (1),  $\alpha$ -purple bacteria (2), mitochondrial homologues (3), spirochaetes, chlamydia and *P. gingivalis* (4), chloroplasts and cyanobacteria (5) and Gram-positive bacteria (6).

**FIGURE 4.10. Hsp60 phylogenetic tree incorporating *B. forsythus* sequence.**



**Figure 4.10.** Molecular phylogenetic tree deduced from analysis of the nucleotide sequences of hsp60 sequences from 9 different species; *Escherichia coli*, *Actinobacillus actinomycetemcomitans*, *Pseudomonas aeruginosa*, *Mycobacterium paratuberculosis*, *Bacteroides forsythus*, *Porphyromonas gingivalis*, *Leptospira interrogans*, *Helicobacter pylori* and human mitochondrial protein P1 (hsp60 homologue). The tree was built by maximum likelihood criteria with the programs Puzzle (Version 4.0.2) and the Tree.View (1.5). The numbers give the quartet puzzling reliability (support values) for the internal branches. The length of the horizontal branches is proportional to the numbers of nucleotide substitutions.

Representative species from different bacterial groups identified by Gupta (Fig. 4.9) were chosen for comparison to *B. forsythus* hsp60. As seen here, the newly identified hsp60 gene is most closely related to the periodontal organism *P. gingivalis* in this tree.

## **CHAPTER 5**

**Expression of *B. forsythus* hsp60 in a prokaryotic vector  
system and purification of recombinant protein**



## **INTRODUCTION**

The hsp60s are widely held to be efficacious stimulators of immune reactions, as evidenced by the accumulating literature that documents hsp60-reactive T and B lymphocytes from patients with a wide range of different diseases and infections (Haregewoin *et al*; 1989; Tsoulfa *et al*, 1989; De Graeff-Meeder *et al*, 1990, 1991; Kaufmann, 1990; Kaufmann *et al*, 1991; Fu *et al*, 1993; Astarloa *et al*; 1996; Coates, 1996; Gruber *et al*, 1996; Krenn *et al*, 1996; Peeling *et al*, 1997; Portig *et al*, 1997; Rudolphi *et al*, 1997; Warr *et al*, 1997; Lemos *et al*, 1998). Some workers have also found that apparently healthy subjects can also carry hsp60-reactive immune components (Munk *et al*, 1988, 1989; Handley *et al*, 1996) but it may be that these antibodies have persisted from a response to a previous infection and are still circulating in detectable amounts. Either way, the human immune potential against hsp60 has yet to be fully determined, as has the significance of this immune response and its possible role in pathogenicity.

Recently, several workers have measured antibody titres to hsp60 in an attempt to define patient sub-populations in large disease groups such as arteriosclerosis and cardiomyopathy. As smaller sub-populations are better characterised, so too can therapy be better targeted, with increased specificity improving efficacy (Gruber *et al*, 1996; Prakken *et al*, 1996; Portig *et al*, 1997). Notably, Schett *et al* (1997) suggested that salivary anti-hsp60 could be used as a diagnostic marker for gingivitis. Testing periodontitis and gingivitis patient groups along with healthy controls, they found that all the groups carried anti-hsp60 antibodies in their sera, but that high concentrations of antibodies against a cross-reactive hsp60 epitope were found only in the sera from

gingivitis patients. As *B. forsythus* has been implicated in particular forms of periodontitis, including recurrent periodontitis (Lai *et al*, 1987; Gersdof *et al*, 1993; Gu *et al*, 1997), it may be that antibodies specific for *B. forsythus* hsp60 could be used diagnostically to predict which patients may be more likely to suffer from recurrent disease. Similar to this idea, Prakken *et al* (1996) have shown that antibodies to hsp60 in the serum of patients with oligoarticular juvenile rheumatoid arthritis predicted disease remission.

Some data exists which indicates that particular strains of *B. forsythus* have developed a level of antibiotic resistance (van Winkelhoff *et al*, 1997). As antibiotic resistance has become an unavoidable evolutionary consequence, albeit one forced by man, it is now accepted that as the use of current antibiotics will inevitably decline, antibacterial drugs will take the form of highly targeted vaccines and other modulators of our own immune response. The hsp60 proteins offer themselves as an attractive candidate target for such developments. Their immunogenicity is well recognised, and their ubiquity and conservation proffers the advantage of mass characterisation which will allow the rapid identity of species-specific epitopes from virtually any infectious bacterial species. A vaccine against such a peptide fraction could be accurately targeted to specific bacterial agents, could produce a rapid and strong immune response and could eliminate a protein known to be essential for cell viability. Alternatively, it has been suggested that a dampening down of our immune responses to hsp60s in localised sites of autoimmune disease may be sufficient to break a cycle of spiralling hsp60 production by human cells and hsp60 antibody attack that results in tissue destruction common to autoimmune diseases (Cohen and Young, 1991). The dampening effect would

be best achieved by modulating the regulators of anti-hsp immune responses. Such regulators could be anti-idiotypic T cells that recognise the antibodies that bind commonly conserved hsp60 epitopes. As anti-idiotypic T cells are already thought to be part of the regulatory process we possess to control anti-hsp responses and prevent them from escalating into systemic autoimmune processes (Cohen and Young, 1991), there would be limited harm in introducing a population of these cells at a localised site of autoimmune disease, and their effect would only be consequential where levels of hsp60 antibody populations were highest.

With this wealth of potential follow-on work, it was a necessary first step to synthesise working amounts of the *B. forsythus* hsp60 to allow preliminary characterisation of the protein. The production of purified *B. forsythus* hsp60 protein may be used to raise antibodies, and to determine regions of the protein homologous to other hsps previously characterised.

There are two main techniques which could be utilised to produce purified *B. forsythus* hsp60 protein. The first technique involves isolating the protein from pure cultures of *B. forsythus* naturally expressing hsp60. Hinode *et al* (1996) detailed a procedure specifically for isolating hsps from periodontopathogenic bacteria. Their technique is based on previous work which has purified hsps via use of their ability to bind substrates such as adenosine 5'-triphosphate (ATP) (Khandekar *et al*, 1993). Hinode's method isolated proteins under denaturing conditions by binding hsp60 from heat-stressed cell cultures to ATP agarose columns and then eluting the protein with low concentration ATP. Other workers who have used this ATP-binding affinity of hsp60 proteins to allow purification used high performance liquid chromatography (HPLC)

systems (Khandekar *et al*, 1993; Jindal *et al*, 1994). This confers the advantage of purifying protein in its native form, but incurs the disadvantage of the high cost of HPLC systems. Hinode succeeded in producing purified hsp60 proteins from *A. actinomycetemcomitans*, *P. gingivalis* and *B. forsythus* without using HPLC, although the yield of *B. forsythus* hsp60 was low. Nakano *et al* (1995) purified the hsp60 protein of *A. actinomycetemcomitans* from a liquid culture of the bacterium using DEAE-sepharose and hydroxyapatite chromatography.

The second method, chosen for this study, is to clone the gene encoding the protein to be expressed into a vector expression system, and then transform this into host cells capable of expressing the protein. Thus eukaryotic cells, for example yeasts, should be used to synthesise protein from the coding sequence of eukaryotic genes, whilst bacterial species such as *E. coli* and *B. subtilis* can be utilised to produce exogenous prokaryotic proteins. Typically, these systems have been engineered such that an element of inducer control is present to switch on production of the foreign gene. The expression vector carries a suitable antibiotic resistance gene to allow the selection of recombinant transformants, and the gene insertion site on the plasmid is proximal to a tag that is co-expressed with the protein to allow purification. Various systems are currently available for the affinity purification of such recombinant proteins. Most rely on the affinity of an immobilised ligand for the tag on the protein of interest. For example, some systems use immobilised antibody ligands or proteinaceous affinity tags such as  $\beta$ -galactosidase (Horner, 1996) to bind and purify recombinant protein from the sample. The disadvantages of having a large proteinaceous tag are that the tag is only

functional in its native form, which stipulates that the protein of interest must be isolated under native conditions. This purifies a protein that retains its correct structure and functional properties, but can be limiting in that over-expressed protein in prokaryotic vector systems often have to be purified under denaturing conditions to increase their solubility. Also, the protein affinity tags can be highly antigenic and can interfere with measures of the purified protein's biological activity. Thus, the tags have to be removed and the protein re-purified before the antigenicity of the protein can be validated.

As an alternative to this, 6x histidine (His) affinity tags have been developed. These protein tags are small, uncharged and non-antigenic, and therefore do not interfere with the activity of the recombinant protein. The 6x His tag can also be used to purify proteins under denaturing conditions. The 6x His system has been used before to isolate hsp60 protein from human mitochondria (Handley *et al*, 1996).

This chapter describes the cloning of *B. forsythus* groEL into an expression vector, and over-expression of the protein product in *E. coli*. The recombinant protein was purified by use of immobilised metal affinity chromatography to a nickel ion resin. Although the yield of purified protein was low, the protein isolated reacted with anti-hsp60 monoclonal antibodies.

## **MATERIALS AND METHODS**

### ***Construction of recombinant plasmid***

The *B. forsythus* hsp60 gene previously generated by PCR (Chapter 3) was cloned into pBAD-TOPO (Invitrogen), a prokaryotic vector system for the expression of PCR products. Expression of this vector in *E. coli* is regulated by arabinose, with expression from the *ara* BAD promoter ( $P_{BAD}$ ) being turned on in the presence of arabinose. Only very low levels of transcription from  $P_{BAD}$  occur in the absence of arabinose.

The cloning site of pBAD-TOPO contains a polyhistidine region which encodes a metal-binding domain. This allows affinity purification of recombinant fusion protein by immobilised metal affinity chromatography (IMAC) using a metal-chelating resin.

The *B. forsythus* hsp60 gene was amplified by PCR using *Taq* DNA polymerase. This enzyme causes the PCR product to have a 3' A overhang, which enabled annealing to the corresponding 3' T overhang on linearised pBAD-TOPO vector. Essentially this permits directional cloning of the PCR product into the vector, such that the 6x histidine tag is attached at the 3' end of the gene (ie at the carboxyl terminus of the protein). The vector was supplied in a kit containing other components required for the reactions. These are the same as those listed in Chapter 3 except that this plasmid was provided with TOP10 One Shot<sup>TM</sup> competent cells for transformation. This strain allows blue/white screening without addition of IPTG to the agar medium.

The protocol for the cloning reactions was the same as for cloning into the pCR2.1-TOPO vector, as described in Chapter 3. Samples of the

transformation reaction were spread on LB-agar plates containing ampicillin, as previously described.

### ***Analysis of recombinant clones***

Small-scale preps of recombinant clones were obtained using the boiling method. Plasmids were analysed by simultaneous digestion of 10µl of the resuspended plasmid prep with restriction enzymes *Bam*HI and *Sac*I. This enabled the orientation of the insert to be determined.

### ***Induction of fusion protein synthesis and optimisation of conditions***

Clones with inserts in the correct orientation were selected. A pilot study was performed to optimise the arabinose concentration required for maximum expression of the recombinant protein. Five tubes containing 10 ml L-broth and 100µg/ml

ampicillin were each inoculated with 0.1ml of an overnight culture of the selected clone. The cultures were incubated in an orbital shaker at 37°C and 200rpm until they attained an OD<sub>600</sub> of 0.5.

During this time four 10-fold serial dilutions of a 20% arabinose stock solution (provided in the pBAD-TOPO kit) were prepared in dH<sub>2</sub>O.

When the cultures had reached an OD<sub>600</sub>=0.5, a 1ml aliquot of sample was taken from each tube, pelleted by centrifugation (14,000 rpm, 30 sec), and the pellet stored at -20°C. These samples were the zero time points in the study.

The series of arabinose dilutions was then added to the tubes of culture as follows:

#### *Arabinose dilution series*

Tube	Stock solution of arabinose	Vol. added (ml) to 9ml culture	Final conc. Of Arabinose
1	0.002%	0.09	0.00002%
2	0.02%	0.09	0.0002%
3	0.2%	0.09	0.002%
4	2.0%	0.09	0.02%
5	20.0%	0.09	0.2%

The cultures were mixed with the inducer and incubated at 37°C with shaking for 4 hours, after which time 1ml samples were taken and pelleted and stored as for the zero time point samples.

#### ***Preparation of samples***

Prior to analysis by SDS-PAGE (see below), the zero hour and 4 hour samples for each arabinose concentration were prepared by resuspending each pellet in 100µl 1X SDS sample buffer (Table 5.1). Each sample was incubated on a 95°C heating block for 5 min and 10µl samples loaded onto the SDS gel for electrophoresis. The remainder of the samples were stored at -20°C.

#### ***Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)***

SDS-PAGE gels were composed of a 12% acrylamide separating gel and 4%



stacking gel. Components of the gel mixes are shown in Table 5.1. SDS-PAGE was performed on a vertical Mini-Protean® II Dual Slab Cell (Bio-Rad) following the manufacturer's instructions. The separating gel was poured to a level 1cm below the bottom of the well-forming comb, and overlaid with a layer of 100% isopropanol to smooth the upper surface of the liquid gel. The gel was left to polymerise for 1 hour, and the isopropanol then rinsed off with distilled water. The stacking gel was poured on top of the separating gel, and the well forming comb put into place. This was left to polymerise for 1 hour before the gel was installed in the buffer chamber. Gels were electrophoresed at 200V in 1X running buffer for 45 min, until the dye front of the samples reached the bottom of the gel. On completion of electrophoresis the unit was dismantled and the gel stained with Coomassie Blue (Table 5.1) for 1 hour. The gel was then destained (Table 5.1) for 2 hours, until the background staining was reduced sufficiently for bands to be clearly visible.

### ***Purification of fusion products using ProBond™ resin***

The six histidine residues expressed at the carboxyl terminus of the recombinant protein have a high affinity for ProBond™ resin (Invitrogen). Once bound, the protein could be eluted from the resin by washing with an imidazole pH gradient under native conditions. Protein was purified following a protocol outlined by the resin manufacturers in "Xpress™ System Protein Purification". Other buffers required for the procedure were made as detailed in Table 5.2.

#### ***i) Preparation and pre-equilibration of ProBond™ columns***

The resin was fully resuspended by inverting and tapping the bottle, and 2 ml

applied to a 5ml centrifuge tube. The resin was packed in the tube by centrifuging at 800Xg for 2 min in a MSE benchtop centrifuge.

The buffer was aspirated without disturbing the packed resin, and the resin resuspended in 7ml sterile water. Resin was packed by centrifugation as before. The water was aspirated and the resin washed 3 times in 7ml Native Binding Buffer (Table 5.2) by resuspending and centrifuging as previously described.

## ***ii) Preparation of bacterial cell lysates and sample application***

A 50ml culture of transformed bacteria carrying the plasmid bearing the recombinant protein was prepared by inoculating 50ml L-broth (containing 100µg/ml ampicillin) with 50µl glycerol stock of bacteria. This was incubated overnight at 37°C whilst shaking at 200 rpm. The following day, the optical density of the culture was measured and adjusted to an OD<sub>600</sub>=0.5. The culture was then induced with the optimum concentration of arabinose, which had previously been determined as 0.02% arabinose (final concentration). The culture was incubated for a further four hours. The cells were then harvested by centrifugation at 5000rpm for 5 min in a Sorvall SS-34 rotor. The pellet was resuspended in 10ml Native Binding Buffer (Table 5.2), egg white lysozyme added to 100µg/ml, and the sample incubated on ice for 15 min. The cells were lysed by sonication and rapidly freeze-thawed 3 times by alternately freezing the sample at -80°C and then warming at 37°C. Insoluble debris was removed from the lysate by centrifuging at 3000Xg for 15 min, and the clear supernatant maintained at -20°C until use. The protein to be purified from the lysate was bound to the resin by resuspending the pre-equilibrated resin with

two 5ml aliquots of the lysate. The resin was resuspended in 5ml lysate and gently rocked for 10 min to prevent the resin packing. The sample was centrifuged at 800Xg, supernatant removed and the resin resuspended in the remaining 5ml lysate as previously described.

### ***iii) Column washing and protein elution under native conditions***

The resin (with bound protein) was washed three times with 4ml Native Binding Buffer by resuspending the resin in the buffer, rocking gently for 2 min and then centrifuging at 800Xg for 2 min to separate the resin from the supernatant. The resin was then washed three times with 4ml Native Wash Buffer (Table 5.2) in the same way.

To elute the bound protein, the resin was sequentially resuspended in elution buffers of increasing imidazole concentration. The resin/buffer mix was rocked for 5 min prior to centrifugation, and the supernatants stored at -20°C. Prior to SDS-PAGE analysis, the samples were concentrated in an Amplicon unit. 5ml samples were reduced to ~0.5ml.

### ***Protein purification using TALON™ Metal Affinity Resin***

TALON resin (Clontech, UK) is a cobalt-based IMAC resin for the purification of 6x His proteins. A protocol utilising TALONspin columns to isolate recombinant protein under denaturing conditions was followed.

### ***i) Sample preparation***

A 25ml culture of pBAD-TOPO carrying the *B. forsythus* hsp60 gene was established. The culture was induced with 0.02% (final concentration)

arabinose as previously described.

Cells were harvested by centrifuging at 2000Xg for 15 min at 4°C. The isolated pellet was resuspended in 2ml lysis buffer (Table 5.3), stirred and sonicated until the viscosity of the suspension was reduced. The sample was centrifuged at 10,000Xg for 20 min to pellet insoluble material. The supernatant, which was cloudy but not opaque, was transferred to a clean tube.

### ***ii) TALONspin column purification***

A TALONspin column was held upright, and flicked until the resin settled in the bottom of the column. A breakaway seal at the bottom of the column was snapped off, and the column placed in a 1.5ml microcentrifuge tube. The clear top lid of the column was removed, and the tubes spun in a microcentrifuge at 700Xg for 2 min. The column was removed from the centrifuge tube, and a cap placed over the nozzle at the bottom of the column. 1ml of the cell preparation was applied to the column, and once the resin bed was thoroughly wet (after 30 sec) the contents were briefly vortex-mixed to resuspend the resin, and gently rocked for 5 min to allow the 6x His protein to bind to the resin. Both caps were removed from the column, and then centrifuged as before. The eluate was discarded. The cap was replaced on the bottom nozzle, and 1ml of pH 7.0 wash buffer (Table 5.3) applied. The resin was soaked by the wash buffer, and resuspended and gently rocked as previously. Both caps were removed again, and the column centrifuged at 700Xg for 2min. This wash was then repeated.

Once it had been ascertained that the resin was semi-dry after centrifugation, the bottom nozzle was capped and 500µl elution buffer containing 20mM

PIPES applied (Table 5.3). The resin bed was soaked for 1 min in the elution buffer, prior to the resin being resuspended by briefly vortexing. Both column caps were removed, and the column placed in a clean 1.5ml microcentrifuge tube. This was centrifuged at 700Xg for 2 min, and the eluate retained. The elution step was repeated with another 500 $\mu$ l of elution buffer. The resulting 1ml of purified protein was stored at -20°C until analysed by SDS-PAGE and Western blotting.

### ***Western blotting***

Following SDS-PAGE of samples, six pieces of Whatman 3MM paper and a piece of 0.45 $\mu$ m nitrocellulose filter paper were cut to the same size as the gel. It was important that the papers and gel were all of the same size to prevent short-circuiting of the electro-blotting apparatus. The Whatman paper and nitrocellulose membrane were equilibrated in transfer buffer for 5 min at room temp. The top left corner of the nitrocellulose was marked with a soft lead pencil to allow for later orientation of the blot.

On a clean, flat surface, three pieces of the wet Whatman paper were stacked, and the gel carefully placed on top of this pile. It was ensured that there were no air-pockets between the gel and the paper. The nitrocellulose membrane was layered on top of the gel, and the membrane gently rolled over with a sterile 10ml pipette to remove air-pockets. The remaining three pieces of Whatman paper were put on top of the pile.

The entire 'gel sandwich' was placed onto the graphite plate of an electro-transfer unit. The electrode cassette was assembled by placing another graphite plate on top of the gel sandwich. Electro-transfer was performed at

low voltage (1-10V), 40mA for 90 min. After this time, the apparatus was disconnected and the gel sandwich carefully peeled apart. By using pre-stained molecular weight markers, it could instantly be ascertained that transfer had taken place successfully when the markers could be seen on the nitrocellulose membrane.

### ***Blotting procedure***

All washes took place by agitating on a rocking platform at room temp. Buffers and solutions used throughout this procedure are listed in Table 5.4.

1. The nitrocellulose filter was placed in a clean glass dish and blocked with blocking buffer for 30 min.
2. The blocking buffer was poured off, and the nitrocellulose washed in washing solution for 10 min.
3. Primary antibody (anti-groEL, IgG raised in rabbit) was diluted as required in blocking buffer. Typically 15ml of antibody solution was applied. The primary antibody was allowed to bind for 2 hours.
4. The antibody solution was poured off. The filter was rinsed with 3x10 min washes of 15ml washing solution.
5. The filter was transferred to a clean dish. Secondary antibody (anti-rabbit IgG), diluted in 15ml blocking buffer, was applied for 2 hours.
6. Secondary antibody was poured off, and the filter washed as at step 4.
7. Bound antibody was detected using freshly made detection solution (15ml). The filter was left in the detection solution in a dark place for 30-45 min.
8. The reaction was stopped by washing the filter in water for 5 min. The filter was then blotted dry and stored in a sealed plastic bag.

**Table 5.1: SDS-PAGE stock solutions, gel mixes and buffers**

*1.5M Tris-HCl, pH 8.8*

Tris base	27.23g
dH <sub>2</sub> O	to 150ml
Adjust to pH 8.8 with 1N HCl	

*0.5M Tris-HCl, pH 6.8*

Tris base	6g
dH <sub>2</sub> O	to 100ml
Adjust to pH 6.8 with 1N HCl	

*10% sodium dodecyl sulphate (SDS)*

SDS	10g
dH <sub>2</sub> O	to 100ml

*12% Separating gel*

30% acrylamide/bis-acrylamide	4.0ml
1.5M Tris-HCl pH 8.8	2.5ml
10% SDS	100µl
dH <sub>2</sub> O	3.35ml
10% APS (freshly prepared)	50µl
TEMED	5µl

***Table 5.1 (continued)***

***4% Stacking gel***

30% acrylamide/bis-acrylamide	1.3ml
0.5M Tris-HCl pH 6.8	2.5ml
10% SDS	100 $\mu$ l
dH <sub>2</sub> O	6.1ml
10% APS (freshly prepared)	50 $\mu$ l
TEMED	10 $\mu$ l

***5X Running buffer stock***

Tris base	3.75g
glycine	18.0g
SDS	1.25g
dH <sub>2</sub> O	to 250ml

***Sample buffer***

0.5M Tris-Hcl pH 6.8	1.0ml
glycerol	0.8ml
10% SDS	1.6ml
$\beta$ mercaptoethanol	0.4ml
0.05% (w/v) bromophenol blue	0.2ml
dH <sub>2</sub> O	4.0ml



**Table 5.1 (continued)**

*Coomassie blue*

Coomassie blue R-250	0.5g
methanol	200ml
acetic acid	50ml
dH <sub>2</sub> O	to 500ml

*Destain*

methanol	200ml
glacial acetic acid	50 ml
dH <sub>2</sub> O	to 500ml

**Table 5.2: Pro Bond™ resin purification reagents and buffers**

*Stock solution A (10X)*

monobasic sodium phosphate (NaH <sub>2</sub> PO <sub>4</sub> )	27.6g
sodium chloride	292.2g
dH <sub>2</sub> O	to 1 litre

*Stock solution B (10X)*

dibasic sodium phosphate	28.4g
sodium chloride	292.2g
dH <sub>2</sub> O	to 1 litre

**Table 5.2 (continued)**

*3M Imidazole stock buffer (10X)*

imidazole	20.6g
stock solution A	8.77ml
stock solution B	1.23ml
dH <sub>2</sub> O	to 100ml

Adjust to pH 6.0 with HCl or NaOH as necessary.

*Native Binding Buffer*

1X Stock solution A	2.9ml
1X Stock solution B	47.1ml

Adjust to pH 7.8 using 1X Stock solution A to lower and 1X Stock solution B to raise the pH.

*Native Wash Buffer*

1X Stock solution A	37ml
1X Stock solution B	13ml

Adjust to pH 6.0 using 1X Stock solution A to lower and 1X Stock solution B to raise the pH.

**Table 5.2 (continued)**

*Imidazole Elution Buffers*

Four imidazole gradient elution buffers were formed by combining volumes of Native Wash Buffer and 3M Imidazole Stock Buffer as below:

	3M Imidazole	Wash Buffer
50mM	0.08ml	4.92ml
200mM	0.33ml	4.67ml
350mM	0.58ml	4.42ml
500mM	0.83ml	4.17ml

**Table 5.3: TALONspin column purification buffers**

*Lysis buffer pH8.0*

Reagent	Final concentration	Amount for 50ml sol <sup>n</sup> .
NaH <sub>2</sub> PO <sub>4</sub> (pH 8.0)	50mM	0.35g
Tris-HCl (pH 8.0)	10mM	0.5ml
Urea	8M	24g
NaCl	100mM	0.29g

Adjust final pH to 8.0

**Table 5.3 (continued)**

*Wash buffer*

Reagent	Final concentration	Amount for 50ml sol <sup>n</sup> .
NaH <sub>2</sub> PO <sub>4</sub>	50mM	0.35g
Urea	8M	24g
NaCl	100mM	0.29g

Adjust final pH to 7.0

*Elution buffer*

Reagent	Final concentration	Amount for 50ml sol <sup>n</sup> .
NaH <sub>2</sub> PO <sub>4</sub>	50mM	0.35g
Urea	8M	24g
PIPES	20mM	0.30g
NaCl	100mM	0.29g

Adjust final pH to 6.0

**Table 5.4: Western blot buffers and solutions**

*Transfer buffer*

Reagent	Final concentration	Amount for 1litre sol <sup>n</sup>
Tris-HCl (pH 8.0)	25mM	3.0g
Glycine	192mM	14.4g
Methanol	20%	200ml

Adjust final pH to 8.3.

**Table 5.4 (continued)**

**TNT**

Reagent	Final concentration	Amount for 1 litre sol <sup>n</sup>
Tris-HCl (pH 8.0)	10mM	10ml
NaCl	150mM	8.76g
Tween 20	0.05%	0.5ml

**Blocking buffer**

3% bovine serum albumin (BSA) in TNT

**Washing solution**

0.1% BSA in TNT

**Detection solution**

66µl nitroblue tetrazolium salt (NBT) (75mg/ml stock) and 50µl 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate, BCIP solution) (50mg/ml stock) in 15ml 0.1M Tris-HCl.

**Antibodies**

Anti-GroEL, IgG raised in rabbit (Sigma-Aldrich) (used at 1:10,000 dilution).

Anti-rabbit IgG, raised in goat, alkaline phosphatase conjugate (Sigma-Aldrich) (used at 1:10,000 dilution).

## **RESULTS**

### ***Construction, structure and mini-prep analysis of recombinant plasmids***

The structure of the pBAD-TOPO plasmid used to express the *B. forsythus* hsp60 protein as a fusion with a 6x His tag is shown in Fig 5.1. The resulting recombinant plasmid is referred to as pBAD*Bf*.

Mini-prep digestion analysis of recombinant plasmids determined the orientation of the hsp60 gene insert. The fragment sizes resulting from *Bam*HI and *Sac*I digestion of a recombinant plasmid with the gene in the correct orientation were predicted to be 5.4-kb, 0.2-kb and 0.1-kb. As the two smaller fragments would be too small to be visualised on an agarose gel, the digest products would essentially appear as one band. A gene inserted incorrectly would yield digestion fragments of 4.1kb, 1.5kb and 0.1kb, and this would be seen on the gel as two distinct bands. Restriction endonuclease digestion with *Eco*RI indicated that all the clones examined carried the hsp60 gene insert in the correct orientation for expression of the *B.forsythus* hsp60 protein.

### ***Arabinose-induced expression of the fusion protein***

The fusion protein product of pBAD*Bf* was expected to be approximately 60kDa, as the 6x His tag was not of sufficient size to contribute significantly to the molecular weight of the fusion protein.

Expression of pBAD*Bf* was subject to regulation by the inducer arabinose. In the absence of arabinose, expression from the *ara* BAD promoter occurs at very low levels (illustrated in Fig 5.2). The arabinose concentration needed for maximum expression of the vector was determined in an optimisation study (Fig 5.3). Following incubation with varying concentrations of arabinose,

pBADBf cultures were processed and analysed via SDS-PAGE and staining of the proteins contained in the gel. This confirmed that there was only very low expression of the fusion protein in the absence of arabinose. When arabinose concentrations were increased, synthesis of a ~60kDa protein increased proportionally. The optimised conditions required for maximal expression of the induced protein were found to be when the culture was incubated in the presence of 0.02% arabinose for 4 hours at 37°C (Fig 5.3). Levels of arabinose higher than this lead to a corresponding decrease in protein yield.

#### ***Verification of the identity of the induced protein by Western blotting***

To confirm that the inducible, over-expressed protein identified in the expression study was the hsp60 protein, SDS-PAGE gels were repeated as above in duplicate. One gel was stained with Coomassie blue to verify the presence of the inducible protein. The other gel was Western blotted using a commercially available anti-GroEL (anti-hsp60) antibody as the primary detection antibody. As this antibody had been raised in rabbits, anti-rabbit IgG antibody was used as the secondary antibody that would bind to the primary antibody. Protein bands reactive with the anti-GroEL antibody can be seen in Fig 5.4, which correspond to the over-induced protein band seen on Coomassie blue stained gels.

A strongly labelled band was observed in the induced samples, which has an apparent molecular weight of 62-kDa. A much weaker band of similar size was also detected in the uninduced samples. It is most likely that this is the endogenous *E. coli* hsp60 which will be present in the host *E. coli* TOP10 strain. The *E. coli* hsp60 is expressed constitutively and would undoubtedly be

immunoreactive with the anti-GroEL antibody used in the screening process.

### ***Purification of recombinant fusion protein using ProBond™ columns***

Preliminary examination of protein purified using the ProBond columns

indicated that it was necessary to concentrate the protein samples using an Amplicon unit before protein bands could be seen to bind anti-groEL.

However, even after the protein had been concentrated bands were still not very strong, as can be seen in Fig 5.5. The double banding pattern indicates that the hsp60 protein has either been co-purified with another, smaller molecular weight protein, or that the purified hsp60 has undergone some proteolysis resulting in the smaller fragment. The latter explanation is the likely cause of the secondary band, as it is also able to bind anti-groEL antibody. With the protein concentration so low, an alternative purification technique using TALONspin columns was utilised in an attempt to improve the yield.

### ***Purification of recombinant fusion protein using TALONspin columns***

*B. forsythus* hsp60 purified from TALONspin columns can be seen bound to anti-groEL in Fig 5.6. As seen in this figure, the molecular weight of the purified protein appears to be higher than was predicted, at approximately 80kd. However, the ability of the purified protein to bind hsp60 specific antibodies and the inducibility of this protein was taken as assurance of its identity. The optimum imidazole concentration for eluting the protein was 200mM. However, as with the ProBond columns, the yield was still low.



## DISCUSSION

When the whole cell lysates of induced cells expressing the recombinant *B. forsythus* hsp60 protein were blotted with anti-hsp60 antibody, it was apparent that the antibody was capable of binding to a range of protein bands smaller than the induced 62-kDa protein (Fig 5.4). Reviewing the available literature, similar multiple protein banding patterns have previously been observed with anti-hsp antibodies. Thole *et al* (1988) noted that the *Mycobacterium bovis* 65-kDa antigen appeared to be partially degraded to low molecular weight products when this protein was expressed in *E. coli*. This phenomenon was also recorded by a group working with hsp60 of *Chlamydia trachomata* (Horner, 1996), and it is possible that a similar situation arises when the hsp60 protein of *B. forsythus* is expressed in *E. coli*. Even monoclonal antibodies raised against groEL homologues, which by definition are specific for only this antigen, have been seen to frequently cross-react with low molecular weight proteins or degraded protein fractions, resulting in the detection of several bands (Thole *et al*, 1988; Maeda *et al*, 1994; Ando *et al*, 1995; Nakano *et al*, 1995). Young *et al* (1987) observed that monoclonal antibodies to the 65-kDa antigen of *M. leprae* frequently bound to bands of various molecular weights in Western blots. They suggested that the most likely origin of the multiple banding is that an original protein, ie. the 65-kDa hsp, undergoes progressive proteolytic digestion to produce multiple protein fragments, each possessing a subset of the total antibody binding sites of the intact protein. As they observed that antibodies against N-terminal epitopes bound the smaller weight fragments more readily than antibodies to C-terminal epitopes, it appears that the initial site of proteolytic cleavage is near the N-terminus of the hsp.

Another factor to consider is the purity of the anti-GroEL preparation. The preparation used in this study was the polyclonal IgG fraction of rabbit antiserum which was formulated by repeated inoculation of rabbits with recombinant *E. coli* hsp60. Presumably, the rabbit's immune system had had previous assaults by *E. coli* and would have raised antibodies to other *E. coli* antigens. These other antibodies would be present in the IgG fraction of the rabbit serum, and could be reacting with the endogenous *E. coli* antigens present in the cell lysates on the SDS gel. This problem may be partially overcome by pre-absorbing the anti-GroEL with *E. coli*, although this was not an option available in the current study as pre-absorption of the anti-groEL with disrupted *E. coli*, to reduce background binding, would simultaneously prevent any reactivity of the anti-hsp antibodies with *B. forsythus* hsp60.

The proteolysis of cellular proteins by bacteria that express digestive enzymes is a recognised problem with regards to the interpretation of SDS-PAGE analysis of whole cell proteins (Weidner *et al*, 1996). Proteolysis of protein samples prior to SDS electrophoresis can result in greatly varying banding patterns within species, the diversity of which cannot be attributed to differences in bacterial strains or growth conditions alone. If proteolysis of samples is extensive, high molecular weight bands can disappear and diffuse smears in the low molecular weight part of the gel may materialise. Proteolysis of cellular proteins can occur once bacterial cells are solubilised in SDS sample buffer. Treatment of cells with trichloroacetic acid or low pH buffer (pH<4) prior to SDS analysis can prevent degradation of cellular proteins. Alternatively, specific protease inhibitors can be used to treat cells if it is desired that the proteins should not be denatured. Weidner (1996) also

suggested that the proteolytic inhibitor be used during protein purification procedures or cell fractionation.

Proteolysis of the expressed *B. forsythus* hsp60 recombinant by endogenous *E. coli* proteases may offer some explanation as to why recombinant *B. forsythus* hsp60 was only purified in small amounts. As mentioned above, Young *et al* (1987) had evidence to suggest that proteolysis of hsp60 molecules was initiated at the N-terminal end of the molecule. An alternative explanation is that a cloning anomaly occurred which altered the reading frame of the *B. forsythus* hsp60 gene. However, it is unlikely that such a chance occurrence is the explanation for the poor protein yield seen in this study, as SDS-PAGE analysis of the recombinant expression products shows an inducible gene of the correct size (Fig 5.3).

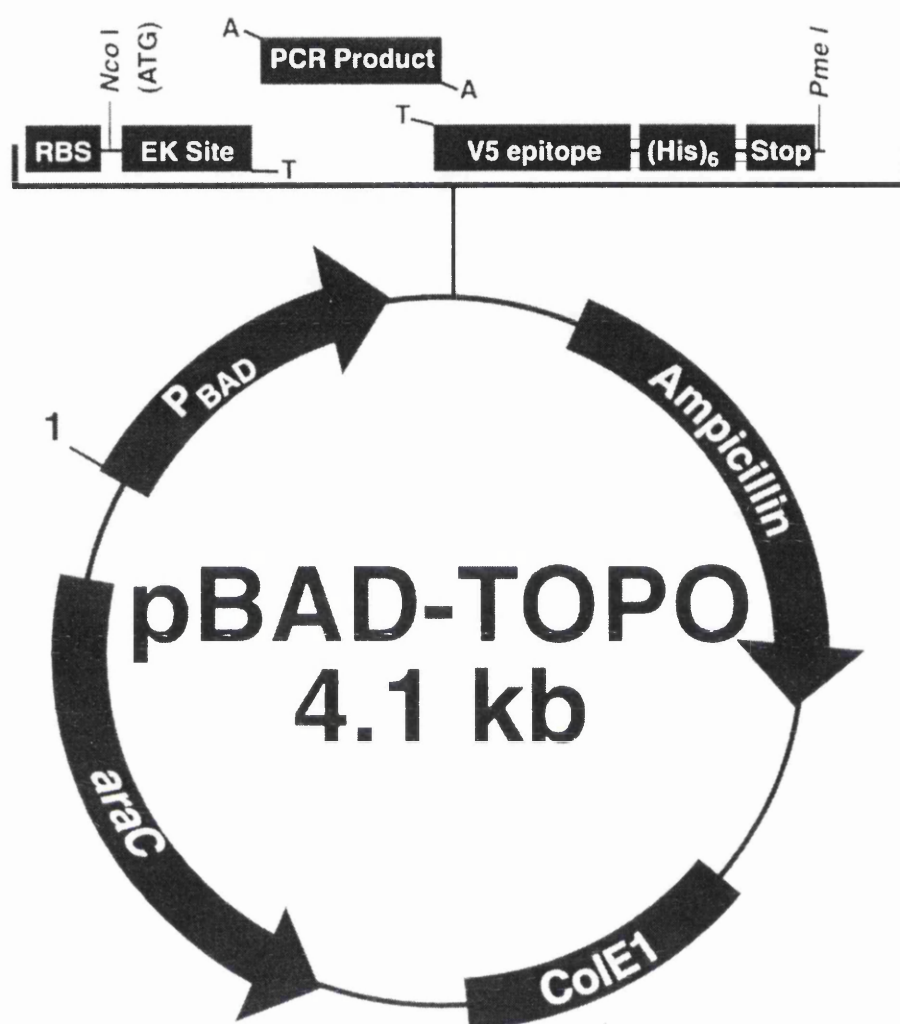
Another potential problem may be the nature of the protein that attempts were made to isolate. Hsp molecules, due to their function as protein chaperones, naturally have a high binding affinity for a diverse range of cellular proteins. The hsp60 of *B. forsythus*, once expressed in *E. coli*, may be of sufficient similarity to endogenous *E. coli* hsp60 to recognise and bind other *E. coli* proteins. This would increase the size, and possibly the structural formation and solubility of the recombinant *B. forsythus* hsp60, and the protein would not be able to be purified using the procedures outlined in this chapter.

As it appeared that some small amount of purified protein was being purified, it would seem that the protein was being co-expressed in the correct reading frame with the 6x His tag, but that a post-transcriptional event was taking place which hindered isolation of higher yields. This could either be due to proteolysis of the recombinant protein that removed the tag, in which case the

recombinant hsp60 would not be able to bind to the affinity chromatography resin, or due to it binding an endogenous *E. coli* protein causing the *B. forsythus* hsp60 to adopt a non-soluble format that is incompatible with the purification procedures used here.

As the protein was isolated in small amounts, it was decided that there was an insufficient amount of the purified recombinant available to advocate testing the antigenicity of the protein by the ELISA method. The protein also appeared to have co-purified with at least one other protein, thus conferring no advantage to using the purified preparations over whole cell lysates. It was therefore decided to proceed with the antigenicity studies that had been planned for purified *B. forsythus* hsp60 by screening whole cell lysates of induced transformants over-expressing *B. forsythus* hsp60 by the Western blotting method. This is described in Chapter 6.

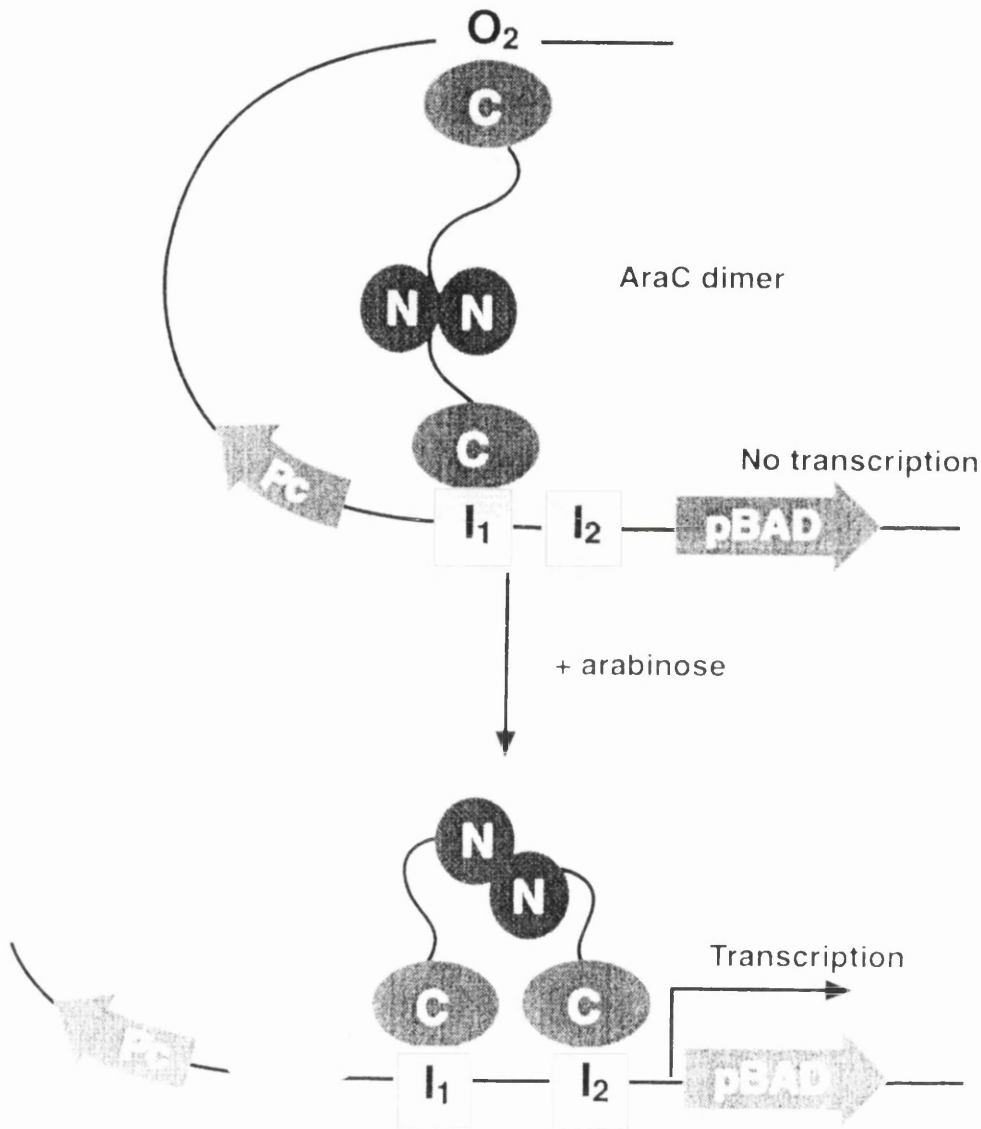
FIGURE 5.1. Plasmid map and features of pBAD-TOPO.



**Figure 5.1.** Plasmid map and features of pBAD-TOPO. The map above shows features of the plasmid pBAD-TOPO which permits five-minute cloning of *Taq* polymerase amplified PCR products for regulated expression in *E. coli*. The metal binding domain encoded by the polyhistidine tag ((His)<sub>6</sub>) allows purification of recombinant protein by immobilised metal affinity chromatography.

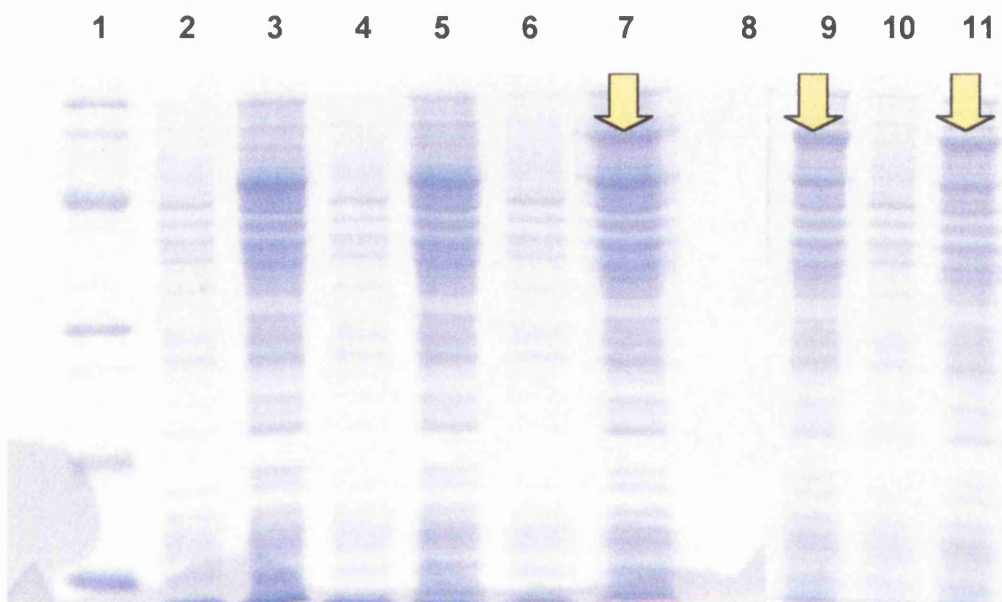
### FIGURE 5.2. Protein expression regulated by arabinose.

Protein expression regulated by arabinose. In the presence of arabinose expression from  $P_{BAD}$  is turned on while the absence of arabinose permits only low levels of transcription from the  $P_{BAD}$  promoter. Briefly, the regulatory functions as described below:



The *araBAD* promoter used in pBAD-TOPO is both positively and negatively regulated by the product of the *araC* gene, a transcriptional regulator. In the absence of arabinose the AraC dimer contacts the  $O_2$  and  $I_1$  sites of the *araBAD* operon, forming a DNA loop which prevents transcription. When arabinose is supplied, arabinose forms a complex with AraC and causes the regulator to release the  $O_2$  site due to a conformational change of the regulator, and binds the  $I_2$  site adjacent to the  $I_1$  site instead. This relaxes the DNA loop and permits transcription (Ogden *et al*, 1980).

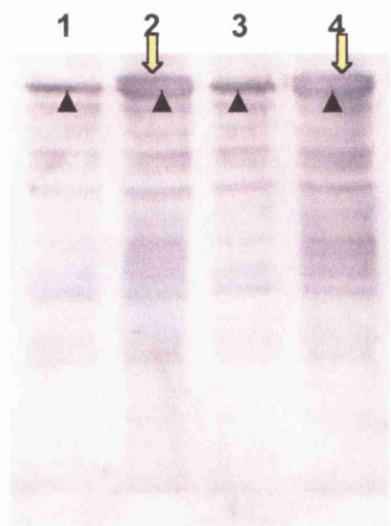
**FIGURE 5.3. Arabinose induction of recombinant *B. forsythus* hsp60 expression.**



**Figure 5.3.** SDS-PAGE gel stained with Coomassie blue. Arabinose induction of recombinant *B. forsythus* hsp60 expression. Molecular weight marker\* (lane 1), recombinant culture induced with **0.00002%** arabinose, t=0 (lane 2), induced with 0.00002% arabinose, t=4 hours (lane 3), induced with **0.0002%** arabinose, t=0 (lane 4), induced with 0.0002% arabinose, t=4 hours (lane 5), induced with **0.002%** arabinose, t=0 (lane 6), induced with 0.002% arabinose, t=4 hours (lane 7), induced with **0.02%** arabinose, t=0 (lane 8), induced with 0.02% arabinose, t=4 hours (lane 9), induced with **0.2%** arabinose, t=0 (lane 10), induced with 0.2% arabinose, t=4 hours (lane 11). The expression of a novel protein can be seen as the arabinose level increases (marked with the yellow arrows). The optimum arabinose concentration for expression of this inducible protein was taken to be 0.02% (lane 9), as this concentration produced the strongest inducible protein band as compared to other concentrations of arabinose.

\*107-, 76-, 52-, 33-, 27-, 19-kDa.

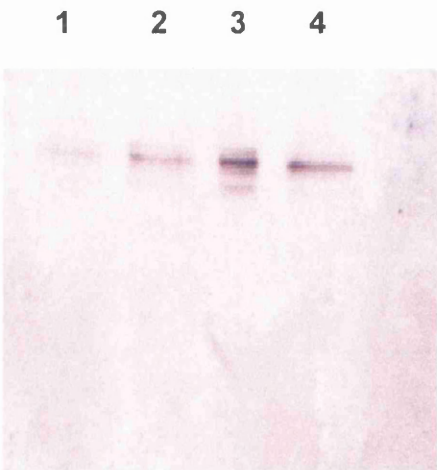
**FIGURE 5.4.** Expression of recombinant *B. forsythus* hsp60, Southern blotted with anti-groEL.



**Figure 5.4.** Recombinant protein expression was induced with arabinose, and electrophoresed proteins transferred to hybridisation membrane. Transferred protein was probed with commercially available anti-groEL (diluted 1:10,000). Recombinant *B. f.* hsp60 in *E. coli* TOP10 induced with 0.02% arabinose, t=0 (lane 1), recombinant *B. f.* hsp60 induced with 0.02% arabinose, t=4 hours (lane 2), recombinant *B. f.* hsp60 induced with 0.2% arabinose, t=0 (lane 3), recombinant *B.f.* hsp60 induced with 0.2% arabinose, t=4 hours (lane 4). An induced protein reactive with anti-groEL can be seen in lanes 2 and 4 (yellow arrows). Endogenous *E. coli* hsp60 has also been stained in all lanes (black arrows).

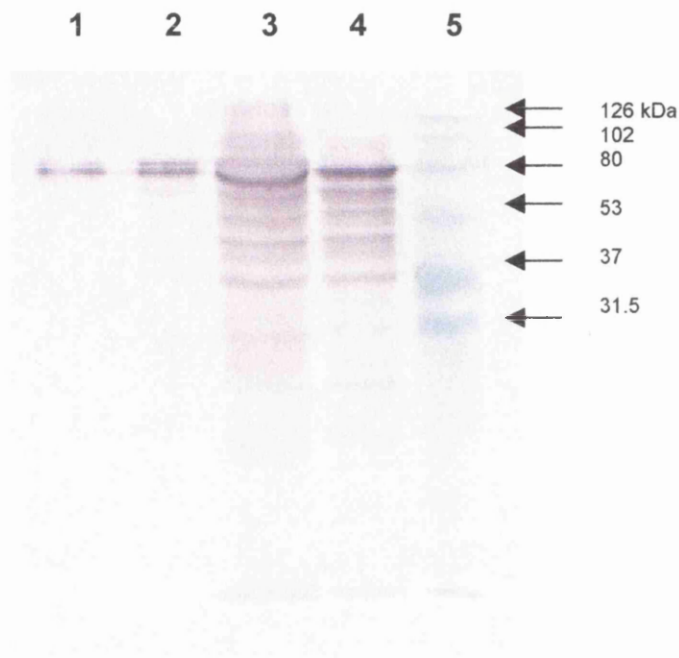


**FIGURE 5.5.** Recombinant *B. forsythus* hsp60 purified with ProBond resin and eluted with imidazole.



**Figure 5.5.** ProBond purified recombinant *B. forsythus* hsp60 protein was transferred to hybridisation membrane and probed with commercially available anti-groEL (diluted 1:10,000). Recombinant protein eluted with 500mM imidazole (lane 1), eluted with 350mM imidazole (lane 2), eluted with 200mM imidazole (lane 3), eluted with 50mM imidazole (lane 4).

**FIGURE 5.6.** Recombinant *B. forsythus* hsp60 purified using TALON prep.



**Figure 5.6.** TALON purified protein was transferred to hybridisation membrane following electrophoresis and probed with commercially available anti-groEL (diluted 1:10,000).

TALON purified recombinant protein from arabinose induced culture (lanes 1 and 2), recombinant *B. forsythus* induced with 0.02% arbinose, t=4 hours (lane 3), recombinant *B.f.* hsp60 induced with 0.02% arabinose, t=0 (lane 4). Pre-stained molecular weight markers in lane 5 are indicated.

## **CHAPTER 6**

**Immunoreactivity of *B. forsythus* hsp60 tested against  
healthy and chronic adult periodontitis serum**

## INTRODUCTION

Many bacterial hsp60s have been shown to be immunogenic proteins, including the groEL homologues of *Salmonella typhi* (Tang *et al*, 1997; Panchanathan *et al*, 1998), *Streptococcus pyogenes* (Lemos *et al*, 1998), *Helicobacter pylori* (Amini *et al*, 1996), *Campylobacter jejuni* (Wu *et al*, 1994), *Chlamydia trachomatis* (Domeika *et al*, 1998; Peeling *et al*, 1997; Taylor *et al*, 1990) and *Mycobacterium* species (Buchanan *et al*, 1987; Debruyne *et al*, 1987; Kolk *et al*, 1984; Thole *et al*, 1988). Hsp60s are clearly the targets of the host immune response in a broad range of infections, which may be related to the abundance of these proteins expressed in cells subject to stress. Increased synthesis of hsps following stress frequently incurs a drop in total cell synthesis of up to 40% (Lemos *et al*, 1998). Buchmeier and Heffron (1990) showed that the groEL homologue is one of the most abundant proteins expressed by *Salmonella typhimurium* in infected macrophages, and as the macrophage is a major antigen-presenting cell this may contribute to the immunodominance of hsps of pathogenic bacteria.

The significant immunoreactivity of hsp60s and their possible role in infectious diseases has been substantiated by studies which detected the presence of antibodies against bacterial hsp60 in the sera of affected patients. Antibodies against a heat-inducible 65 kDa protein of *S. pyogenes* were detected in the sera of patients with rheumatic fever, mild streptococcal disease, systemic lupus erythematosus (SLE) and rheumatoid arthritis, but not in the serum from healthy control subjects (Lemos *et al*, 1998). The anti-streptococcal hsp60 antibodies are thought to possess a high level of species specificity, due to the fact that the serum that recognised streptococcal cell lysates was unable to bind

any protein from *E. coli* cell lysates. This was unusual, as it is generally assumed that most samples of human serum will bear antibodies to *E. coli* that has been previously encountered by the host. The observation of a specific anti-streptococcal hsp60 response by Lemos is partially verified by a previous study by Bahr *et al* (1990), which found that sera from patients with rheumatic fever showed no significant binding to hsp60 of *E. coli*, *M. tuberculosis* or human cells.

The sera of patients with typhoid fever has been shown to be reactive with the hsp60 antigen of *S. typhi* (Tange *et al*, 1997). Heat-induced whole-cell extracts of *S. typhi* were immunoblotted with sera from 12 patients with typhoid fever, and nine samples bound 58-, 68- and 88-kDa hsps. None of 10 healthy serum samples recognised the hsps. *E. coli* groEL monoclonal antibodies also recognised the induced *S. typhi* protein, suggesting that the *S. typhi* and *E. coli* groEL homologues share antigenic epitopes.

Autoantibodies to human hsp60 have also been reported for a range of diseases, including dilated cardiac myopathy (Portig *et al*, 1997) rheumatoid arthritis (Boog *et al*, 1992; DeGraeff-Meeder *et al*, 1990; Kaufmann *et al*, 1990) and atherosclerosis (Xu *et al*, 1993). It has been suggested that the autoantibodies to human hsp60 are often cross-reactive and may have been initially raised against an infectious agent such as *E. coli* (Handley *et al*, 1996), mycobacteria (Gruber *et al*, 1996) or *C. trachomatis* (Yi *et al*, 1993).

With regards to periodontitis, serum and salivary antibodies have been found to be reactive with hsp60 proteins of bacterial origin. Levels of anti-hsp60 salivary antibodies were significantly raised in gingivitis patients as compared to healthy and periodontitis samples, whereas serum antibody levels against hsp60 were

raised in periodontitis patients (Schett *et al*, 1997). Antibody titres to hsp60 of *P. gingivalis* and human hsp60 have been shown to be elevated as compared to healthy serum samples (Ando *et al*, 1995; Maeda *et al*, 1994; Tabeta *et al*, 1999).

Antibodies to hsp60 antigens may contribute to the pathogenesis of disease by a number of different means. As stress proteins are known to reside on the surface of resting or activated cells (Jarjour *et al*, 1990; Kaufmann, 1994; Soltys and Gupta, 1996), anti-hsp60 antibodies may have a direct cytotoxic effect on stressed cells, sustaining inflammation of localised tissue. Anti-hsp60 antibodies may also disrupt the physiological function of chaperonins and render cells more vulnerable to hostile environmental conditions. Such a scenario was demonstrated by Riabowol *et al* (1988) when heat shock was shown to be lethal to fibroblasts micro-injected with antibodies against hsp70. Stress proteins are thought to be presented to healthy individuals constantly, in order to maintain a homeostatic regulation of the immune response against hsps (Cohen, 1992).

This idea is supported by the finding that naturally processed peptides presented by human gene products are homologous to endogenous stress proteins (Jardetzky *et al*, 1991). Interference of this MHC presentation of peptides by hsp-specific antibodies may disrupt the homeostasis and lead to autoimmune disease.

Some data exists which argues against the level and significance of serum antibody levels to both human and bacterial hsp60s (Jarjour *et al*, 1991) and discrepancies are reported between studies examining similar subjects (Latif *et al*, 1993; Portig *et al*, 1997). The results of the immunoblot assays discussed in this chapter can be influenced by a number of variables, such as choice of

antigenic substrate, amount of antigen analysed per lane on SDS gels and the amount of antigen successfully transferred to nitrocellulose prior to blotting, dilution concentration and length of incubation of both primary and secondary antibodies used in the immunoblots, and methods used to visualise reactive protein bands. As these variables are not constant between different studies, the interpretation of any immunoblot results and the ensuing inferences derived from the results should be viewed with an awareness of these study inconsistencies.

This study was designed to investigate the potential reactivity of IgG antibodies in sera from patients with rapidly progressive periodontitis against the hsp60 of *B. forsythus*. It was decided to measure this IgG response as significantly elevated levels of IgG antibodies against the main periodontal pathogens have been reported in all forms of periodontitis (Murayama *et al*, 1988). One-dimensional immunoblotting was used to detect the anti-hsp60 antibodies, a procedure deemed as one of the most sensitive screening methods available (Stott, 1989). Lysates of induced *E. coli* TOP10 carrying the plasmid pBADBf, which expresses the recombinant hsp60 protein of *B. forsythus*, were used as the antigenic substrates in immunoblots. Although this substrate would naturally be contaminated with the endogenous hsp60 of *E. coli*, the hsp60 of *B. forsythus*, by virtue of its over-expression, is easily distinguishable from the *E. coli* homologue as the strongest band visualised on SDS-PAGE gels.

## **MATERIALS AND METHODS**

### ***BCA protein assay***

#### *i) Samples*

Cultures of *E. coli* TOP10 harbouring the plasmid pBADBf, which contained the *B. forsythus* hsp60 gene cloned in-frame into the pBAD TOPO expression plasmid, were incubated in L-broth with 0.1mg/ml ampicillin at 37°C and 200 rpm in an orbital incubator until an OD<sub>600</sub> of 0.5 was attained. 0.02% arabinose was added to half the culture and incubated as before for a further 3hr 30 min. The remainder of the culture was incubated similarly in the absence of an inducer. Following this, a 1ml sample from both cultures was pelleted in a benchtop microcentrifuge for 30 sec, and the pellet then resuspended in 100µl distilled water. 2µl, 5µl, and 10µl samples from each were removed for assay, and the measurements plotted against the protein standard curve to determine the protein concentration in the samples, and to ensure that each serum sample was used to screen a controlled and constant amount of protein.

#### *ii) BSA standard curve*

A solution of 2mg/ml BSA was prepared by solubilising 0.2g BSA in 100ml distilled water. The A and B reagents of the Bicinchoninic Acid (BCA) protein assay were mixed at a 50:1 ratio to make 20ml. 1ml of this reagent pre-mix was mixed with the following volumes of BSA solution to result in an increasing range of final protein concentrations:



Vol A:B pre-mix	1ml	1ml	1ml	1ml	1ml	1ml	1ml	1ml	1ml
Vol BSA solution	0	0.5 $\mu$ l	1 $\mu$ l	2 $\mu$ l	4 $\mu$ l	6 $\mu$ l	8 $\mu$ l	10 $\mu$ l	20 $\mu$ l
Final protein concentration	0	1 $\mu$ g	2 $\mu$ g	4 $\mu$ g	8 $\mu$ g	12 $\mu$ g	16 $\mu$ g	20 $\mu$ g	40 $\mu$ g

At the same time, the A:B pre-mix was mixed with samples of the induced and uninduced cultures as below:

Vol. A:B pre-mix	1ml	1ml	1ml
Vol. sample	2 $\mu$ l	5 $\mu$ l	10 $\mu$ l

All samples, including those for the BSA standard curve, were incubated at 60°C for 30 min and the OD<sub>562</sub> measured. The OD measurements from the BSA samples were plotted against the final concentration of each sample, and a standard curve drawn through the points. For those OD values from the test culture samples that were found to lie on the curve, the protein concentration could be determined.

### **SDS-PAGE**

SDS gels comprising of a 4% stacking gel with a 12% lower acrylamide gel were used in a Mini-protean ® II Dual Slab cell (Bio-Rad), as described previously.

Induced samples of pBADBf to be screened with patient serum were adjusted to

OD<sub>600</sub>=0.5. 1ml of sample was pelleted in a benchtop microcentrifuge for 30 sec. The pellet was resuspended in 100µl sample buffer and heated at 95°C for 5 min. 10µl of this suspension was loaded per lane, which corresponded to approximately 40µg protein per lane.

### ***Western blotting***

After electrophoresis of the induced whole cell fraction, proteins were transferred to nitrocellulose membrane as previously described. Following transfer, the nitrocellulose was cut into strips of one-lane width with a sterile scalpel so that each lane could be screened with a different serum sample. Each induced culture sample strip was Western blotted following the procedure detailed in Chapter 5. The primary antibody was serum collected from healthy or diseased patients, and was used at a 1:500 dilution in 5ml blocking buffer (Table 5.4). The secondary antibody was an anti-human IgG alkaline phosphatase conjugate, and was used at a 1:10.000 dilution in a 5ml volume. Serum samples and secondary antibody were both incubated with individual strips for 2 hours. Bound secondary antibody was detected with the same chromogenic substrate mix as described in Chapter 5 (Table 5.4).

### ***Serum samples***

#### ***i) Patient samples***

Patients included in the study had no medical contra-indications and had not received dental treatment or antibiotics in the previous six months. An initial dental examination then followed which consisted of a full mouth periodontal charting using a Brodonic constant pressure probe (25g) and a radiographic

examination with orthopantomograms. Only those patients diagnosed as suffering from chronic adult periodontitis were included in the study.

A control group consisting of 13 healthy subjects were matched for age ( $\pm 5$  yrs), sex and race. The subjects demonstrated no clinical evidence of existing or previous periodontal disease. On clinical examination there was less than 20% bleeding on probing and no periodontal pocket depths greater than 3mm. Subjects with systemic disease, pregnancy, long term medication or antibiotics in the 6 months prior to examination were excluded. Serum was collected and assigned a code number in the same manner as for the experimental patients.

#### *ii) Serum collection*

Approximately 30ml of blood was taken. Blood was collected into glass tubes using the Vacutainer system. The sera was separated by coagulating the samples and centrifuging at 3000 rev/min (Centaur 2, MSE centrifuge) for 5 min. Sera samples were coded and stored at  $-40^{\circ}$  until required.

Patient recruitment, clinical examination and characterisation and sera collection was kindly performed by Dr. A. Smith.

#### *iii) Pre-absorbing sera*

Serum was pre-absorbed with disrupted whole *E. coli* TOP10 cells to reduce the level of background banding on the blots that would result from patient antibodies recognising and binding *E. coli* proteins. The cells were disrupted by adding 30% w/v glass beads prior to shaking the cultures in a disintegrator for 30 min. The glass beads were then removed from the cell suspension by brief centrifugation and the supernatant was removed. Serum diluted 1:500 in 5ml

blocking buffer was pre-absorbed with 750 $\mu$ l disrupted cells at room temp for 2 hours.

## **RESULTS**

### **BCA assay**

The standard protein concentration curve derived from the BCA assay was compiled, and using OD<sub>562</sub> readings taken for uninduced and induced whole cell samples of pBADBf in TOP10 that had been similarly digested with assay reagents, the protein concentration of these samples could be determined from the graph. The uninduced samples contained protein at approximately 1mg/ml and induced samples had a higher protein concentration of 4mg/ml.

### **Western blots**

Serum samples from 13 healthy controls and 11 patients with chronic adult periodontitis were analysed. The Western blots of serum reacted with whole cell fractions of induced pBADBf in *E. coli* TOP 10 are shown in Fig 6.1. Of the serum samples taken from patients with rapidly progressive periodontitis, two samples reacted with the induced protein *B. forsythus* hsp60. The induced protein was identified by its apparent molecular weight, migrating as a 60-kDa protein as compared to molecular weight standards. The identification of the reactive proteins as induced *B. forsythus* hsp60 was verified in both cases by blotting an uninduced sample with the same serum samples that yielded the stained bands at 60-kDa. As the example shows, the protein was not detected by 'reactive' serum in the uninduced sample (Fig 6.2, lane 2).

To determine whether the patient antibodies that recognised the *B. forsythus* hsp60 were cross-reactive, a sample of patient serum that had been shown in Fig 6.1 to bind the induced protein was pre-absorbed with disrupted *E. coli*. This served to reduce the level of cross-reactive binding. As can be seen, the pre-

absorbed serum did not bind the induced protein (Fig 6.2, lane 3). This indicated that the patient antibodies that were able to bind the induced *B. forsythus* protein were cross-reactive with *E. coli* hsp60. It is probable that either the antibodies were originally raised against *E. coli* and are able to recognise homologous sequences of the *B. forsythus* protein, or conversely, *E. coli* proteins were recognised by antibodies raised against *B. forsythus*. In both situations, the pre-adsorption of serum with *E. coli* hsp60 would result in the reduced ability of the hsp60 serum antibodies to bind *B. forsythus* hsp60. Fig 6.3 demonstrates the ability of healthy human serum samples to bind *B. forsythus* hsp60. As can be seen, one of the 13 healthy samples carried antibodies reactive with the groEL gene product of *B. forsythus*. This study has shown that components of the human immune response raised against a cross-reactive epitope expressed by *B. forsythus* hsp60 are present in healthy and adult periodontitis patient serum samples.

## DISCUSSION

This chapter documents the immunoreactivity of the groEL protein of *B. forsythus*, which has been ascertained to further define the role of *B. forsythus* hsp60 as a pathogenicity factor in periodontal disease. The antigenicity of this protein was determined by screening whole cell extracts of arabinose-induced *E. coli* TOP10 transformed with pBADBf by means of Western blotting. Electrophoresed cell extracts were screened with human sera collected from 11 patients with chronic adult periodontitis, and 13 clinically healthy subjects. The main finding of this study was the demonstration that sera samples from both patient groups contained antibodies of the IgG class against *B. forsythus* hsp60. Anti-*B. forsythus* hsp60 antibodies were apparent in two out of 11 periodontitis patient samples, and in one out of 13 healthy samples. That the antibodies were primarily binding the over-expressed *B. forsythus* recombinant protein, and not endogenous *E. coli* hsp60, was verified by screening a *B. forsythus* hsp60 positive serum sample from a diseased patient against an uninduced lysate of *E. coli* carrying the pBADBf plasmid as antigenic substrate. This resulted in an antibody-binding pattern that lacked the 60-kDa band. Furthermore, the antibodies against *B. forsythus* hsp60 carried by one patient were demonstrated to be cross-reactive, possibly raised against *E. coli* hsp60, by pre-absorbing the serum sample with disintegrated whole cell fractions of *E. coli*. Using pre-absorbed serum resulted in a binding pattern that again displayed no antibody binding to a 60-kDa protein (Fig 6.2). *B. forsythus* hsp60 has therefore been shown to be an immunogenic protein, against which some periodontitis patients and healthy individuals possess an IgG antibody response. With the size of the patient groups used in this study a

significant difference in the antibody response between the diseased and control groups cannot be interpreted, although this study provides a preliminary indication that an antibody response to *B. forsythus* hsp60 is not unique to patients with chronic adult periodontitis.

Similar to this study, Maeda *et al* (1994) performed an immunological analysis of recombinant *P. gingivalis* hsp60 by Western blotting, and found reactive antibodies to be present in sera from periodontitis patients and in healthy controls. However, another report on recombinant *P. gingivalis* hsp60 documents no response to *P. gingivalis* hsp60 in healthy serum samples whilst finding a high percentage of periodontitis sufferers to be carrying *P. gingivalis* hsp60 antibodies (Tabeta *et al*, 1999). A report on *A. actinomycetemcomitans* hsp60 also found an antibody response in periodontitis patients, but none in healthy controls (Koga *et al*, 1993). As none of these studies use patient groups larger than 20, it is difficult to assess the significance of these early results. A harder task yet will be to understand the role of an immune response to bacterial and human hsp60 in the pathogenesis of periodontitis. A level of antibody reactivity to human hsp60 in periodontitis gingival tissue has been reported (Ando *et al*, 1995; Tabeta *et al*, 1999) which could be a part of an autoimmune process, but whether this process is the healthy removal of infected host cells or the deleterious destruction of human tissue would be hard to prove. It may be that the anti-human hsp60 response is not pathogenic, but that hsp60 antibodies have a pathogenic effect on a tissue-specific antigen, such as periodontal ligament fibroblasts which have been shown to share a level of homology with hsp60 (Gemmell and Seymour, 1995). Alternatively, bacterial hsp60, rather than antibodies to it, may be a causative agent of bone



resorption, as suggested by Kirby *et al* (1995), and further work investigating such aspects of these proteins remains to be done.

It is suggested that the identification of periodontitis patients with antibodies to hsp60 of periodontopathogenic bacteria may be of diagnostic value, and could highlight those patients who may benefit from specific anti-inflammatory treatment (Schett *et al*, 1997; Hinode *et al*, 1998).

Cross-reactive and species-specific antigenic regions of hsp60 homologues have been previously identified, with most work focused on characterising the antigenic determinants of the 65-kDa protein of *Mycobacterium* species (Ivanyi *et al*, 1983; Kolk *et al*, 1984; Mehra *et al*, 1986; Buchanan *et al*, 1987; Young *et al*, 1987; Anderson *et al*, 1988). The first report of the DNA sequence of an hsp60 homologue, the 65-kDa antigen of *M. leprae*, also reports a technique utilised to determine the amino acid sequences of epitopes recognised by *M. leprae* monoclonal antibodies (Mehra *et al*, 1986). After sequencing the entire *M. leprae* hsp65 gene, a sub-library containing fragments of the gene, formed by partial DNA digestion with DNase I, was constructed in a  $\lambda$  bacteriophage vector. Recombinant clones forming the library were screened with *M. leprae* monoclonal antibodies to select those clones which expressed a fragment of epitope-coding sequence. The nucleotide sequence contained within reactive clones was then determined and mapped to the available sequence of the entire protein. Using this technique, six different antigenic determinants of the *M. leprae* 65kDa protein were identified, one of which was unique to *M. leprae*. A study following this work on immunogenic epitopes of hsp60 homologues continued to use the same monoclonal antibodies which recognised the 65-kDa protein of *M. leprae*, in addition with others to make a panel of 23 antibodies

(Buchanan *et al*, 1987). 14 of these monoclonal antibodies were found to recognise distinct epitopes, as proven by cross-competition assays. Of these monoclonal antibodies, only one recognised an epitope unique to *M. leprae*, whereas all the others displayed a greater or lesser amount of cross-reactivity by binding anything from eight to 23 different mycobacterial species. This prompted closer examination of the epitopes involved on the mycobacterial 65-kDa protein, such as the study by Anderson *et al* (1988), which further characterised the monoclonal antibody binding sites. Anderson and colleagues synthesised peptide fragments of the 65-kDa antigen to elucidate which portions of the sequence were recognised by the monoclonal antibodies. Rather than construct small overlapping peptides to cover the sequence of the entire protein, they chose to synthesise fragments that corresponded to the 11 most pronounced hydrophilic peaks of the predicted amino acid sequence of the protein, as it was anticipated that these regions would likely be antigenic. By use of this procedure, 10 epitopes were characterised which bound the monoclonal antibodies, and seven of these epitopes occurred in hydrophilic domains. An *M. leprae*-unique epitope was identified (III E9) possessing the peptide sequence KLKLTGDEA. Since the analogous sequence of the 65-kDa protein from *M. tuberculosis* is ELKLEDGEA, it would appear that specific residue changes at a single site (in this instance a threonine substitution for lysine) can inter-convert an apparently well-conserved epitope into a uniquely specific one. This was also shown to be the situation with an epitope specific for *Neisseria* species (Pannekoek *et al*, 1995) in which a *Neisseria*-specific antibody domain differed from the homologous sequence of *M. tuberculosis* at only one residue.

Of the mycobacterial hsp60 monoclonal antibodies that were identified as cross-reactive (Anderson *et al*, 1988), antibodies F67.13, IIIC8, Y1.2 and IIH9 bound synthetic mycobacterial peptide sequences that were well-conserved and homologous to the corresponding sequence of the *B. forsythus* hsp60 antigen. This can be seen in Fig 6.4, where the peptide sequences which bound mycobacterial hsp60 monoclonal antibodies are aligned with the corresponding sequence from *E. coli* and *B. forsythus*. The epitopes that appear to be best conserved are reactive with antibodies IIH9 and F67.13. Although the reactive sites for antibodies Y1.2 and IIC8 look less well conserved, it has been demonstrated that both these antibodies are cross-reactive with the *E. coli* sequence (Young *et al*, 1987) and would presumably be able to bind the *B. forsythus* homologue as well. The portion of the *B. forsythus* sequence that antibody IIIC8 would theoretically bind has already been identified as a region of the protein that is hydrophilic, and is predicted to be a surface-expressed antigen of the hsp60 protein (Discussion, Chapter 4), which further supports the notion that cross-reactive monoclonal antibody IIIC8 will recognise the *B. forsythus* sequence as well as that of *E. coli* and *Mycobacteria*. This again indicates that the amino acid sequence of an epitope is not always intrinsic for antibody binding, rather, the presence of perhaps only a few crucial residues facilitates antibody recognition of a binding site.

The sequence illustrated in Fig 6.4 shows regions of the *B. forsythus* hsp60 sequence predicted to be hydrophilic which also have a high probability of being surface-expressed peptides, and as such are likely to be antigenic (predictions made using the PEPLOT function of the GCG computer program package; see Chapter 4). As has been shown previously, there are limits as to how

broadly cross-reactive some cross-reactive antibodies are. Thus some mycobacterial cross-reactive antibodies recognise eight different mycobacterial species whilst others recognise 23 different species (Buchanan *et al*, 1987). Likewise, it is plausible that there could be antibodies in periodontitis patient serum that recognise those regions of the *B. forsythus* sequence predicted to be antigenic, but that these antibodies would not recognise mycobacterial hsp65 domains, and therefore have not been identified or characterised previously. Certainly some of the most well-conserved and potentially most antigenic regions of the *B. forsythus* hsp60 sequence and other homologues have not as yet been identified as the antibody binding sites which they may well be.

Several monoclonal antibodies that are raised against mycobacterial species are able to bind the human mitochondrial homologue P1 and conversely, a polyclonal antibody raised against P1 showed some cross-reactivity with cell extracts from *E. coli* and *Salmonella typhimurium* (Dudani and Gupta, 1989). The further ramifications of this are quite apparent with regards to autoimmune reactions. As the mycobacterial antibodies used in Dudani's study are also likely able to bind the *B. forsythus* sequence due to the highly conserved nature of some of their epitopes, it is probable that antibodies that may be raised against these *B. forsythus* epitopes in periodontal disease will also be able to recognise human mitochondrial protein P1. Other workers who have studied hsp60 proteins in periodontal pathogens (Maeda *et al*, 1994; Nakano *et al*, 1995; Hinode *et al*, 1998; Tabeta *et al*, 1999) postulate that cross-reactive antibodies raised against bacterial hsp60 may have a role in the pathogenesis of periodontitis via a mechanism involving autoimmune processes. If regions of *B.*

*for*sythus hsp60 sequence were shown to bind the same cross-reactive antibodies that display such broad cross-reactivity as to recognise both human and mycobacterial antigens, this would go some way to supporting the hypothesis of *B. for*sythus hsp60 antigen being involved in autoimmune processes of periodontitis. However, the human mitochondrial protein is so widely expressed that there must be factors that limit the spread of the autoimmune reactions, i.e. there must be a degree of tissue specificity for this putative autoimmune disease. It may be that the immune reactions against hsp60 are only of pathogenic significance in areas where there are particularly high levels of hsp60 expression, and detrimental autoimmune responses to hsp60 are thus limited to sites of localised inflammation at the periodontium. Alternatively, it may be that hsp60-reactive antibodies are directed against cells or antigens which are periodontium specific, such as the periodontal ligament fibroblast which displays identity with hsp60 proteins (Gemmel and Seymour, 1995).

The characterisation of species-specific epitopes of the hsp60 sequence of different aetiological agents of disease can potentially lead to improved diagnosis of infections and the development of crucially targeted vaccines (Mehra *et al*, 1986; Buchanan *et al*, 1987; Young *et al*, 1987), and will also further extend our knowledge of the aetiology of diseases. It is also important to appreciate which antigenic regions of the protein raise cross-reactive antibodies, as these antibodies may be significant in autoimmune processes.

It is important to note that whilst the majority of studies appear to competently demonstrate the high reactivity of monoclonal antibodies to linear hsp60/65 epitopes, the nature of these studies and the experimental techniques utilised

may bias the characterisation of linear epitopes only, and thus discontinuous epitopes are under-represented in these studies. Firstly, the protein preparations used to immunise mouse B cells for the production of monoclonal antibodies have usually been subjected to some procedure, such as heat-killing of cells or sonication, that results in partially or completely denatured antigen. Thus the laboratory animals are exposed to a large number of continuous epitopes, with many of the discontinuous epitopes disrupted. Secondly, after the animal has raised antibodies, the monoclonal antibodies are frequently identified by their reactivity with 60/65-kDa protein on Western blots. Again, the processing of proteins prior to Western blotting involves steps which are likely to denature the protein to a significant extent, such that any monoclonal antibodies that are detected by Western blots are those which can bind linear epitopes. These factors may account for the high proportion of monoclonal antibodies against hsp60/65 that have specificity for linear epitopes, despite the fact that it has previously been estimated that up to half of all antigenic sites on native proteins are composed of an assembly of primary structure segments of protein that have come together as a result of protein folding to be expressed on the protein surface as discontinuous epitopes, (Berzofsky, 1985; Barlow, 1986). Another possible explanation is that the 60/65-kDa hsps possess structural features that foster binding of antibodies to linear epitopes. This would be the case if the protein naturally adopted an unfolded configuration with continuous epitopes unconcealed on the protein surface. Many of the continuous epitopes currently appreciated appear to reside in hydrophilic peptide sequences, which are also anticipated to be stretches of the protein that are surface expressed. Also, there is some indication that the hsp60 protein is particularly vulnerable to

proteolytic digestion (Young *et al*, 1987), and both these features are in accordance with the hsp60 protein possessing an unfolded structure that expresses a high proportion of linear epitopes.

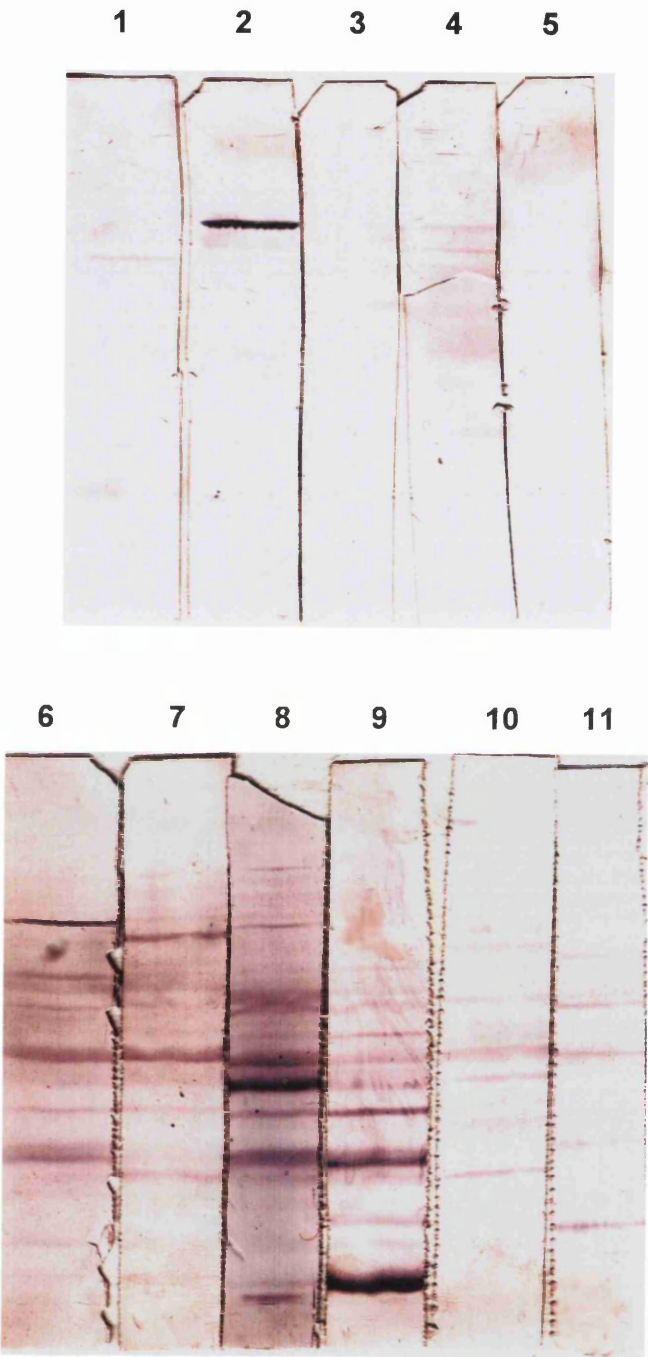
Proposed work to follow the studies presented in this chapter on the initial identification of *B. forsythus* hsp60 as an antigenic protein recognised by antibodies in human serum would be to define the antigenic structure of *B. forsythus* hsp60. This could be achieved utilising a technique which several other groups have successfully employed to distinguish immunodominant epitopes, whereby overlapping peptide sequences are synthesised to represent the entire protein sequence. 9-mer peptides based on the groEL sequence of *S. typhi* were constructed, and the resulting 122 peptides used to coat the surface of polyethylene pins which were then screened with monoclonal antibodies to *E. coli* groEL and with sera from patients suffering from typhoid fever (Panchanathan *et al*, 1998). Three immunodominant epitopes were identified, one of which was recognised both by the sera samples and by an *E. coli* monoclonal antibody. The two other peptide sequences which bound serum antibodies corresponded to a well-conserved region of hsp60 homologues, at amino acid residues 180-205. Continuous B-cell epitopes in *C. trachomatis* hsp60 were identified by synthesising 553 12-mer peptides, that overlapped by all but one residue (Yi *et al*, 1993). The peptides were screened with serum from female patients infected with *C. trachomatis* and 13 major epitopes classified. Seven of the epitopes were matched to sequences of the human hsp60 protein, which had also been synthesised, and were able to bind the serum samples. Interestingly, it was noted that these binding sites did not always share good sequence identity between the bacterial and human

homologue, and that amino acid differences could not be used to predict which epitopes were specific or cross-reactive. A study to characterise antigenic domains of *B. forsythus* hsp60 could be performed by expressing sub-sections of the protein and determining their ability to bind patient serum antibodies to determine where epitopes lie. The sections of the protein to be expressed could be synthesised by constructing a *B. forsythus* hsp60 sub-library from gene fragments. The sequence of the inserts of reactive clones could be determined, and the DNA sequence encoding an epitope attributed to sequences that are shared by multiple antibody-positive recombinant clones. A more efficient method would be to generate hsp60 peptide fragments by PCR, using internal primers selected from the *B. forsythus* hsp60 genomic sequence, and the PCR products then cloned into a suitable expression vector and screened with patient serum or with hsp60 monoclonal antibodies. This method has been previously used to identify epitopes of Neisserial hsp60 (Pannekoek *et al*, 1995). After immunogenic B-cell epitopes for *B. forsythus* hsp60 have been identified, it would be a considerable task to raise monoclonal antibodies to these regions to determine whether the epitopes raise cross-reactive or species-specific antibodies. However, the panel of cross-reactive monoclonal antibodies commercially available could at least identify the conserved epitopes, and perhaps concurrently mark the epitopes likely to be species-specific.

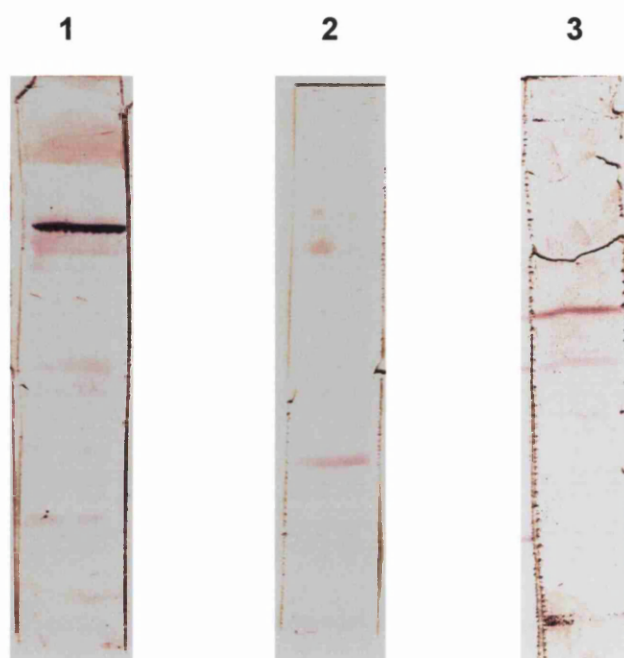


**Figure 6.1.** Western blots demonstrating reactivity of serum from patients with adult periodontitis with recombinant *B. forsythus* hsp60. Electrophoresed whole cell fractions of *B. forsythus* hsp60 expressed in *E. coli* were screened with serum from 11 patients with adult periodontitis. The serum samples contained antibodies that reacted with endogenous *E. coli* proteins, seen as the multiple banding patterns above. Two serum samples, tested in lanes 2 and 6, bear antibodies specific for a protein of approximately 60 kDa which is subject to arabinose control (see fig 6.3.)

**FIGURE 6.1. Western blots demonstrating reactivity of serum from patients with adult periodontitis with recombinant *B. forsythus* hsp60.**

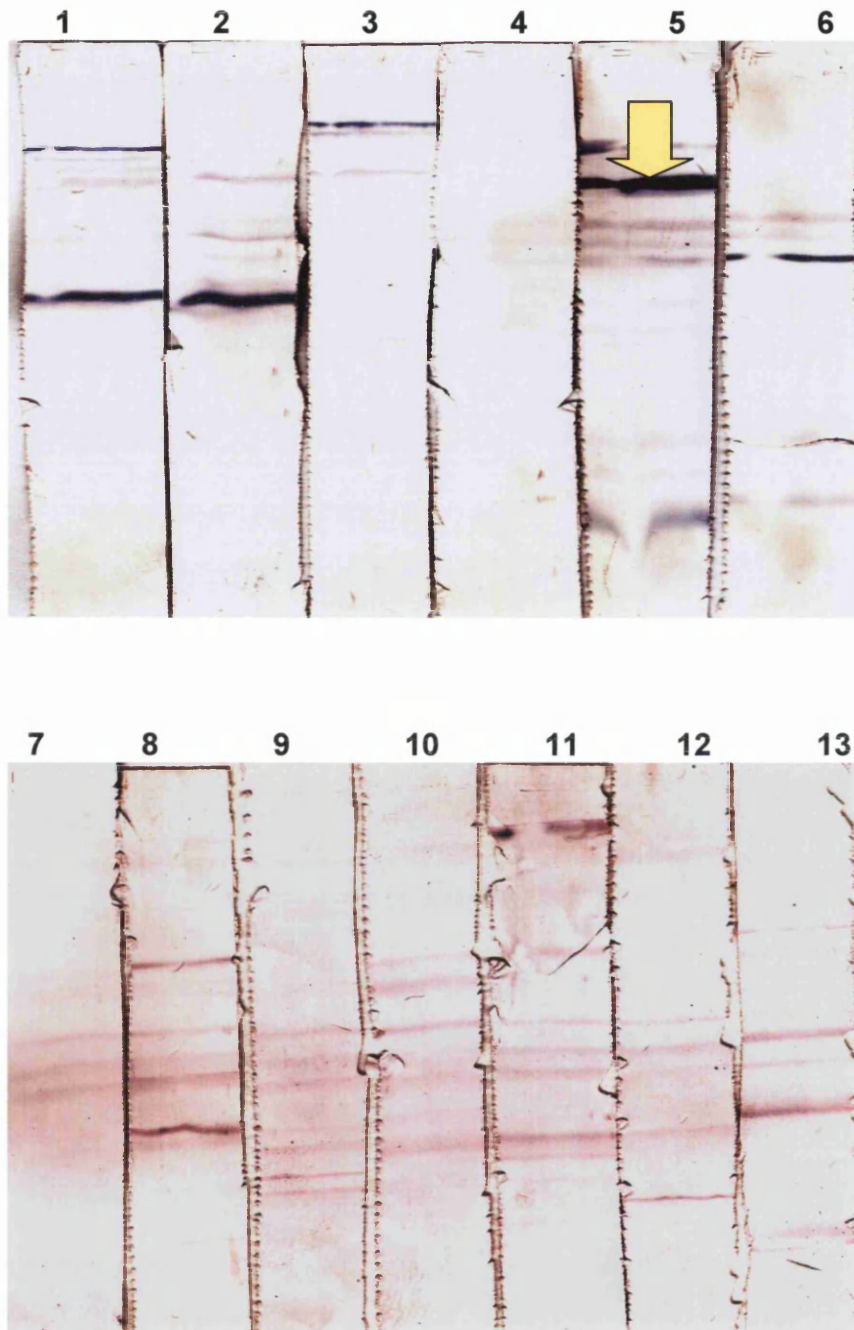


**FIGURE 6.2. Western blot analysis of adult periodontitis serum reactive with recombinant *B. forsythus* hsp60.**



**Figure 6.2.** Western blot analysis of adult periodontitis serum reactive with recombinant *B. forsythus* hsp60. Serum sample bearing antibodies specific for a 60kDa protein (lane 1). The identity of this protein as recombinant *B. forsythus* hsp60 is conformed in lane 2, where the same reactive serum sample is used to blot an non-induced cell fraction. Thus the immunoreactive protein in lane 1 is subject to inducer control. The same serum sample was pre-absorbed with disrupted *E. coli* whole cells and used to screen an induced sample (lane 3). The disappearance of the band detected in lane 1 indicates that the patient antibodies recognising *B. forsythus* hsp60 are cross-reactive with *E. coli* hsp60.

**FIGURE 6.3. Western blots demonstrating reactivity of healthy adult serum samples with recombinant *B. forsythus* hsp60.**



**Figure 6.3.** Western blots demonstrating reactivity of healthy adult serum samples with recombinant *B. forsythus* hsp60. Whole cell fractions of *B. forsythus* hsp60 expressed in *E. coli* were screened with serum extracted from 13 healthy individuals. The serum samples contained antibodies that reacted variably with endogenous *E. coli* proteins. The serum sample in lane 5 has specificity for recombinant *B. forsythus* hsp60 (yellow arrow).

**Figure 6.4.** Conserved monoclonal antibody linear binding sites on hsp60 amino acid sequences. Previously characterised mycobacterial monoclonal antibodies bound synthetic peptide sequences (Anderson *et al*, 1988) which share homology with corresponding sequences of *B. forsythus* (Bf) and *E. coli* (Ec). These monoclonal antibodies have been shown to be cross-reactive with *E. coli* hsp60 (Young *et al*, 1987), and are likely to be able to bind *B. forsythus* hsp60, high-lighting potential antigenic sites of the protein. 1= antibody Y1.2 binding site, 2= IIH9 binding site, 3= F67-13 binding site, 4= IIIC8 binding site (all characterised in Anderson *et al*, 1988).

The boxed sequences are regions of the *B. forsythus* hsp60 sequence predicted to be hydrophilic and surface expressed (Chapter 4) and also therefore potentially antigenic.

**FIGURE 6.4. Conserved monoclonal antibody linear binding sites on hsp60 amino acid sequences**

Bf	-----MAKEIKFDMNARDLLKKGVDELA	23
Ec	-----MAAKDVKFGNDARVKMLRGVNVLA	24
Mb	1.ARRGLERGLNSLA	
Bf	NAVKVTLGPKGRNVILEKKFGAPQITKDGVTVAKEIELACPYENMGAQLV	73
Ec	DAVKVTLGPKGRNVVLDKSFGAPTITKDGVSVAREIELEDKFENMGAQMV	74
Mb	DAVK	
Bf	KEVASKTNDKAGDGTTTATVLAQAIIGVGLKNVTAGANPMDLKRGIKAV	123
Ec	KEVASKANDAAGDGTTTATVLAQAIITEGLKAVAAGMNPMDLKRGIKAV	124
Mb	2.AAGANPLGLKRGIEKA	
Bf	SKVVESIASQSEAVGTNMDRIEHVAKISANGDEGIGKLIAEAMQKVKKEG	173
Ec	TAAVEELKALSVPC-SDSKAIAQVGTISANSDETVGKLIAEAMDKVGKEG	173
Mb		
Bf	VITVEEAKGTETTVEVVEGMQFDRGYISAYFVTDTEKMETQFENPYILIY	223
Ec	VITVEDGTGLQDEL DVVEGMQFDRGYLSPYFINKPETGAVELESPFILLA	223
Mb		
Bf	DKKISVLKDLLPILEQMVQSGRALLIIAEDIDSEALATLVVNRLRGGLKV	273
Ec	DKKISNIREMLPVLEAVAKAGKPLIIAEDVEGEALATAVVNTIRGIVKV	273
Mb		
Bf	CAVKAPGFGDRRKAMLEDIAILTGGTVITEE-KGMKLEDAKMDMLGSADK	322
Ec	AAVKAPGFGDRRKAMLQDIATLTGGTVISEE-IGMELEKATLEDLGQAKR	322
Mb		
Bf	VTVNKDNTTIVKGNQDKAAIESRIGQIKAQIETTTSDYDKEKLQERLAKL	372
Ec	VVINKDTTTIIDGVGEEAAIQGRVAQIRQQIEEATSDYDREKLQERVAKL	372
Mb		
Bf	AGGVAVLYVGAPSEVEMKEKKDRVDDALHATRAAIEEGTVPGGGVAYLRA	422
Ec	AGGVAVIKVGAAIEVEMKEKKARVEDALHATRAAVEEGVVAGGGVALIRV	422
Mb	3.TEVELKERKHRIEDAVRNAK	
Bf	IPALEGLKGENEDETTCIEIVKRAIEEPLRQIVNNAGKEGAVVVQKVKEG	472
Ec	ASKLADLRGQNEQNVGIIKVALRAMEAPLRQIVLNCGEPSVVANTVKGG	472
Mb		
Bf	TGAFGYNARTDVYEDLSEAGVVDPAKVTRIALENAASIAGMFLTTECVVA	522
Ec	DGNYGYNAATEEYGNMIDMGILDPTKVTRSALQYAASVAGLMITTECMVT	522
Mb		
Bf	DKKEEAPAPPMNP-GMGGMGGM	544
Ec	DLPKNDAADLGAAGMGGMGGMGGM	548
Mb	4.DPTGGMGGMDF	

## **CHAPTER 7**

**Construction and screening of a *B. forsythus* genomic library**

## INTRODUCTION

The bacterial population which accumulates at the gingival margin possesses a battery of antigens, some of which are capable of triggering a cascade of host-mediated immune reactions. The antigens of periodontal pathogens are therefore of interest to the researcher for two reasons:

- i) antigens that elicit a strong host response may be protective, and characterisation of these proteins or protein fragments may lead to antimicrobial therapies or vaccine development for improved treatment of periodontitis.
- ii) antigens expressed by periodontal pathogens can induce a chronic and deleterious immune response that results in tissue damage, and as such are directly implicated in the immunopathology of periodontal disease.

Thus as the microbial causative agents of periodontitis continue to be identified, attempts are made to determine which of the multitudinous antigens of the micro-organisms provoke the strongest response from the host, with the aim of further characterising these immunodominant antigens.

Proposed antigens of oral periodontopathic microorganisms have been identified by human antibody responses using sera collected from periodontitis patients. In many cases, the studies go no further than to identify serum-recognised proteins by molecular weight alone. Studying the outer membrane proteins of *A. actinomycetemcomitans*, two groups identified 13 to 15 principal antigens to which human sera reacted ranging in size from 14-78 kDa (Califano *et al*, 1992; Page *et al*, 1992; Watanabe *et al*, 1989). Strains of *A. actinomycetemcomitans* have been differentiated into three serotypes



(Zambon, 1985). Serum antibody levels to the serotype antigens were raised in infected patients. It was noted that each patient was infected with only one serotype, and that juvenile periodontitis patients reacted to serotype antigen b more frequently than to serotype antigens a or c. This would suggest that serotype b of the organism is the most pathogenic, but also highlights the fact that the antibodies a patient carries can be strain- or serotype-specific, and thus patients within the same disease group (i.e. periodontitis patients) may express different antibodies. This makes the task of determining common dominant antigens more difficult. A similar situation has been described for patients infected with *P. gingivalis*, whose antibody response is directed against the outer membrane proteins of this species (Papaioannu *et al*, 1991). Individual patients were shown to have raised antibody levels to one of the three serotypes of *P. gingivalis* (Ebersole and Steffen, 1995). More recently it has also been suggested that more than one serotype of *B. forsythus* exists which may be implicated in periodontal infections and, as has been demonstrated for *A. actinomycetemcomitans* and *P. gingivalis*, different strains appear to affect different patients, which results in serum samples from a range of patients recognising different antigens of the same species (Takemoto *et al*, 1997; Sims *et al*, 1998a and 1998b). With the potential for phenotypic variation between strains it is important to identify which antigens are common to all serotypes if the wider goal of developing treatment strategies for periodontitis is to be achieved.

Patient reactivity to outer membrane proteins has been widely demonstrated (Califano *et al*, 1992; Watanabe *et al*, 1989), but these antigenic determinants have generally remained uncharacterised. The nature of some of these

dominant antigens can be elucidated by pre-absorbing patient serum with a known, characterised antigen and then using this serum to immunoblot a whole cell fraction of a suspected periodontal pathogen. This blot is then compared to the same protein array immuno-blotted with unabsorbed serum. Any band that disappears in the pre-absorbed blot can be assumed to be antigenic and corresponds to the same protein that was used to pre-absorb the serum. This technique has been used to show that lipopolysaccharide (LPS) may be a major antigen of *P. gingivalis* (Schenk, 1985; Boutsis, 1993). LPS is a component of the outer membrane of the cell wall of Gram negative bacteria, and is also referred to as bacterial endotoxin (Stephen and Pietrowski, 1986). The identification of LPS as an important antigen of *P. gingivalis* has been further bolstered by a report indicating that antibodies to *P. gingivalis* LPS are protective and prevent alveolar bone loss in experimental animals immunised with *P. gingivalis* (Vasel *et al*, 1996). Serum IgG antibody levels to LPS of *A. actinomycetemcomitans* have also been shown to be significantly increased in juvenile periodontitis patient groups.

Bacterial fimbriae, also referred to as pili, are deemed virulence factors due to the fact that deletion mutation studies demonstrate that afimbriate mutants are avirulent. *P. gingivalis* mutants which do not express fimbriae have been shown to have a reduced ability to adhere to human gingival tissue (Hamada *et al*, 1994). Western blot studies have demonstrated that serum antibody levels to the fimbriae of *P. gingivalis* are raised in some patients with periodontal disease (Yoshimura *et al*, 1987), indicating that fimbrial proteins can be potent immunogens in some instances of disease.

Various other proteins of periodontal pathogens have been propounded

as dominant antigens. These include assorted proteases that have been best characterised in *P. gingivalis*, although it is likely that this array of enzymes is not unique to *P. gingivalis* (Haffajee and Socransky, 1994). The proteases confer a variety of enzymatic actions, possessing the ability to degrade host structural components such as collagen and fibrinogen (Nitzan *et al*, 1978; Uitto *et al*, 1988; Lawson and Meyer, 1992; Scott *et al*, 1993) and immunoglobulins and complement factors (Sundqvist *et al*, 1985; Grenier *et al*, 1989; Gregory *et al*, 1992). They are crucial to the bacteria for nutrition, adherence and aid survival of the bacterium by degrading opsonins accumulating at the bacterial cell surface. The proteases are evidently important virulence factors, whose foreign nature marks them for recognition by the host humoral immune response. An elevated level of antibodies against them is expected due to their abundance at a site of periodontal infection.

Some Gram negative bacteria express secreted proteins which are bacterial exotoxins of the RTX ('repeats in toxin') family. The family of toxins have in common a glycine-rich tandem repeat in the C-terminal domain of the amino acid sequence. The RTX toxins also differ between species with regards to their target cell specificity. The RTX toxin of *E. coli* has a wide range of cellular targets for example, whereas the *A. actinomycetemcomitans* RTX toxin has a narrow range of targets and affects leukocytes and macrophages of human origin. The toxins damage target cell membranes by forming transmembrane pores, resulting in cell lysis (Stephen and Pietrowski, 1986). The RTX toxin of *A. actinomycetemcomitans* has been shown to be an important virulence factor; >80% of *A. actinomycetemcomitans* isolates from the healthy population

are toxin deficient, whereas 75% of patients with periodontal disease carry RTX+ strains. Elevated antibody levels to the RTX toxin of *A.*

*actinomycetemcomitans* has been repeatedly demonstrated in localised early onset periodontitis patients as compared to healthy controls (Ebersole *et al*, 1983; McArthur *et al*, 1981).

Another group of proteins expressed by periodontal bacteria that are believed to be important immunogens are the heat shock proteins. The immune response to the hsp60s of the periodontal pathogens *A.*

*actinomycetemcomitans*, *P. gingivalis* and *B. forsythus* has been shown to be elevated in patient serum samples as compared to healthy controls (Ando *et al*, 1995; Hinode *et al*, 1998; Tabeta *et al*, 1999). Regardless of whether or not the hsp60s are the triggers of autoimmune responses in periodontal disease as has been suggested, immune reactivity to hsp60 is potentially an important component of the immune response to pathogenic organisms implicated in periodontitis.

In an attempt to identify the protein antigens of *B. forsythus* which elicit a strong antibody response in human periodontitis patients, this chapter describes the construction of a genomic library of *B. forsythus* and the screening of the expression library with serum from a patient previously characterised as being a high responder to *B. forsythus*.

A genomic library ideally contains relatively short lengths of DNA sequences that are representative of an entire genome, such that the complete population of recombinant clones should yield overlapping sequences that span the genome. The cloned fragments should be sufficiently large to carry complete genes and their immediate flanking sequences that may encode promoters.

The size of the inserts is intrinsic to the construction of a good library. The vector is usually the size limiting denominator for fragments in a library, thus if some fragments are too large to insert into the chosen vector, stretches of the genome are not represented in the library and the library has essentially failed. It is equally important that fragments should not be too small either, as this would result in an unmanageable number of clones to analyse and a high proportion of the clones would only carry gene portions from which proteins could not be expressed.

## **MATERIALS AND METHODS**

### ***Construction of the genomic library***

#### ***i) Purification of genomic DNA***

*B. forsythus* genomic DNA was isolated from bacteria as described in Chapter 2.

#### ***ii) Establishing conditions for partial digestion of genomic DNA***

*B. forsythus* chromosomal DNA was partially digested with various concentrations of the restriction endonuclease *Sau3A* to establish the optimum concentration of enzyme required to produce DNA fragments in the size range 2-10 kb. A protocol for this optimisation assay was derived from a previously described method (Glover and Hames, 1995). Serial dilutions of an endonuclease reaction mix were performed to give a range of enzyme concentrations. The reaction mix comprised 10µg *B. forsythus* DNA, 15µl buffer B (Promega), 1.5µl BSA (Promega) and sufficient distilled water to bring the volume to 150µl. Nine tubes were labelled and 30µl of the reaction mix dispensed into tube 1 and 15µl into tubes 2 to 9. 5U *Sau3A* was added to tube 1, mixed thoroughly, and 15µl of this mix added to tube 2. This two-fold serial dilution was repeated through tubes 2 to 8. Tube 9 remained as an undigested control. All tubes were incubated at 37°C for 60 min and the digestion stopped by adding 3µl 0.1M EDTA and 2µl gel loading solution. The same *Sau3A* digests of *B. forsythus* DNA were also constructed as above and incubated at 37°C for 75 min and 90 min. 15µl samples of each digest were electrophoresed on a 0.6% TAE gel and the DNA visualised with UV light.

### ***iii) Partial digest scale-up***

The digestion conditions that yielded the best range of DNA fragment sizes was selected, and the digestion scaled up two-fold. The partially digested DNA was cleaned using a Wizard™ DNA Clean-Up Kit (Promega) following the manufacturer's instructions. DNA was resuspended in 60µl distilled water and 2µl electrophoresed on a 0.8% TBE gel. This served to both quantitate the DNA concentration and to check that the sizes of the fragments generated were in the correct range.

### ***Ligation***

Purified digested DNA was ligated to the vector pUC18 digested with *Bam*HI and dephosphorylated to prevent re-ligation (Amersham Pharmacia Biotech). As the DNA inserts are on average twice the size of the vector, an insert to vector (w/w) ratio of 6:1 was required to give a molar ratio of 3:1, which is the optimal ratio for ligation reactions. Thus 100ng of vector was ligated to 600ng of partially digested *B. forsythus* DNA, using 3U T4 DNA ligase. The ligation was allowed to proceed at 14°C overnight.

### ***Transformation***

2µl of the ligation reaction was used to transform competent *E. coli* TOP10 cells as previously described (Chapter 3). Transformants were spread on L-agar plates containing 0.1mg/ml ampicillin, and plates were incubated overnight at 37°C.

### ***Cloning controls***

The level of background recombination of the cloning vector was ascertained by transforming host *E. coli* TOP10, as previously described, with un-ligated vector. A sample of transformed cells was then plated onto L-agar plates with ampicillin and incubated as described.

### ***Mini-prep analysis of transformant DNA***

24 recombinant colonies were inoculated into 5ml L-broth containing 0.1mg/ml ampicillin and incubated overnight at 37°C. These cultures were screened by mini-prep and *EcoRI* digestion following the procedures described previously (Chapter 2). Digestion with *EcoRI* linearised the recombinant plasmid which could then be analysed by size after electrophoresis.

### ***Immunological screening by colony lift***

This procedure was performed essentially following a protocol previously described (Sambrook *et al*, 1989). To analyse large numbers of clones, 300µl of each transformation was spread on 145mm plates of L-agar containing 0.1mg/ml ampicillin. Master copies of the library were grown on nitrocellulose filters which enabled the library to be transferred onto fresh growth medium to sustain clones. Replicas of the library were made by transferring the colonies via the colony lift technique onto fresh nitrocellulose filters. Generally no more than two replicas could be made from each master plate.

Colonies on the replica plates were lysed to expose internal as well as surface-expressed antigens and screened with the serum of a patient previously characterised as a high responder to *B. forsythus*, as determined by



ELISA. The antiserum used to detect bound patient serum was anti-human IgG alkaline phosphatase conjugate (Sigma). Clones that reacted on the replica filters could be matched to those on the corresponding master plate and recovered for further analysis. Typically, approximately 1000 colonies were obtained on each plate following transformation.

*i) Preparation of master and replica plates*

Nitrocellulose filters were sterilised prior to use, and cut to fit the base of a 145mm petri dish. Each filter was soaked in distilled water and sandwiched between layers of dry 3MM Whatman paper. The stack of filters and Whatman papers was wrapped and sealed in aluminium foil, and autoclaved as liquid (10lb/sq. in). Using sterile blunt forceps, a sterile filter was placed onto a L-agar plate containing ampicillin (100µg/ml). Transformant bacteria were plated onto the filter, the plate inverted and incubated overnight at 37°C. Another filter was dampened by placing it on a fresh agar plate containing ampicillin and IPTG to induce expression of the inserted genes under control of a *lac* promoter (in case the inserted gene did not carry its own promoter). The master filter with grown colonies was removed from its plate and placed colony-side-up on a stack of 3MM Whatman paper. The wet replica filter was carefully placed on top of the master filter and the filters pressed together, with care taken not to move the filters. Using a sterile needle, the filters were pierced around the border in an asymmetrical pattern whilst they were sandwiched together to allow later orientation of the replica plate with the master plate. The filters were gently peeled apart and placed, colony side up, onto fresh plates (the replica plate agar containing IPTG).

Both replica and master plate were incubated at 37°C for several hours until colonies 1-2mm in diameter appeared. After this, the master plate was wrapped in foil and stored at 4°C until the screening results were available. The replica filter was processed for screening as described below.

#### *ii) Processing filters prior to screening*

In a fume hood, the replica filters were lifted from the agar plates and placed onto damp paper towels. The filters were covered with a plastic box and a glass dish containing chloroform placed within the box. The colonies were exposed to chloroform for 15min. After this, the filter was placed in a 145mm petri dish with 15ml lysis buffer (Table 7.1) and rotated at room temp overnight. The filters were then washed in a glass dish with TNT for 2x30 min and then screened.

#### **Serum**

Filters were screened with serum from a patient with adult periodontitis who had previously been characterised as a high responder to *B. forsythus* by ELISA (serum provided and characterised by I. Darby, Glasgow Dental Hospital and School). The serum was pre-absorbed with disrupted *E. coli* and used at a 1:200 dilution. The protocol for screening the filters was the same as that described for Western blotting in Chapter 5.

### ***Pre-absorbed serum***

On a sterile nitrocellulose filter sitting on L-agar, bacterial colonies of the host transformant strain (*E. coli* TOP10) were established. A replica was made as described above and the colonies lysed as for the processing of filters prior to screening. Lysed colonies were incubated with diluted serum (1:500) for 2 hours. The serum was then collected and 0.05% sodium azide added as a preservative. The preabsorbed serum was stored at 4°C until required.

### ***Immunological screening by Western blot***

SDS gels with a 4% upper and 12% lower gel were poured as described in Chapter 4. Transformant colonies to be screened were inoculated into 5ml L-broth with 0.1mg/ml ampicillin and incubated overnight at 37°C with shaking at 200rpm. The cultures were adjusted such that the  $OD_{600} = 0.5$ . 1ml of each adjusted culture was pelleted by centrifuging for 30 sec in a benchtop microcentrifuge. The resulting pellet was resuspended in 100µl sample buffer and heated at 95°C for 5min prior to electrophoresis. 20µl of each sample was loaded onto the gel, with the protein in the sample at a concentration of 1mg/ml as determined by a BCA protein assay (see Chapter 6).

After electrophoresis, the gels were transferred to nitrocellulose membranes as previously described, and blotted with the same high responder patient serum that was used to screen the genomic library by the colony lift technique. The serum was again pre-absorbed with *E. coli* TOP10 whole cells, as described above.

### ***Sequence analysis of recombinant clones***

Recombinant clones carrying inserted DNA sequences of approximately 4-8kb were previously identified by restriction endonuclease digestion analysis. Of these clones, three were selected randomly to be further characterised by sequencing the DNA inserts. Cultures were grown in L-broth with 100µg/ml ampicillin as previously described and 3ml of this overnight culture used to perform a Wizard Plus™ Plasmid Mini Prep, as outlined in Chapter 4. Purified plasmid was used as template DNA for sequencing reactions following a protocol for automated sequencing (Chapter 4). Commercially available consensus primers M13 Forward and M13 Reverse were utilised to generate sequence data. Retrieved sequence data for each reaction was transformed into FASTA format and used to search on-line database sets via use of BLAST searches. The information retrieved from the database sets was preliminary sequence data which was obtained from the Institute for Genomic Research website at <http://www.tigr.org>.

***Table 7.1: Cell lysis buffer***

content:	for 20ml:
100mM Tris	2ml
150mM NaCl	0.18g
5mM MgCl <sub>2</sub>	0.01g
1.5% BSA	0.3g
1µg/ml Dnase	20µg
40mg/ml lysozyme	80µl of a 10mg/ml solution

## **RESULTS**

### ***Partial digestion of *B. forsythus* chromosomal DNA***

*B. forsythus* chromosomal DNA was digested with a range of different enzyme concentrations and for varying incubation times to determine the optimum conditions to yield DNA fragments of 2-10kb, illustrated in Fig 7.1.

### ***Cloning controls***

The level of background recombination of the linearised vector was found to be minimal; a 200µl sample of transformed cells incubated overnight on agar plates yielded less than 30 colonies.

### ***Mini-prep analysis of recombinant clones***

To ascertain the percentage of recombinant clones which contained DNA inserts within the desired size range, randomly selected colonies were subjected to restriction endonuclease digestion to release the inserted fragments, the size of which could be determined by agarose gel electrophoresis. 24 colonies were picked and seeded into 5ml L-broth containing 100µg/ml ampicillin. The cultures were incubated overnight at 37°C with shaking at 200rpm and plasmid DNA extracted using the boiling method described in Chapter 2. Resuspended plasmid DNA was digested with *Bam*HI for three hours, and electrophoresed on a 0.8% agarose gel.

From this analysis, it could be seen that only one in six transformants were carrying a DNA fragment of the desired size (Fig 7.2).

## ***Immunoscreening of the genomic library***

### ***i) Colony lift***

Approximately 10,000 *E. coli* transformants were screened for expression of *B. forsythus* protein antigens using the colony lift technique. Following screening with periodontitis patient serum from a high responder to *B. forsythus*, one clone reacted positively with the serum in duplicate (Fig 7.3). The positive clone was selected from the master plate and a sub-culture grown to be screened once more. However, a preliminary Gram stain of the isolated sub-culture revealed that the organism was a Gram positive coccus, and was therefore a contaminant that had grown on the master plate.

Of all the other plates screened, no positive clones were identified.

### ***ii) Western blot***

To increase the sensitivity of the immunoscreening procedure, transformant colonies were screened by Western blotting to detect the presence of any novel protein expression in transformant *E. coli* clones. Using the original master plates of maintained transformants which had been used for colony lift screening, 98 colonies were selected and screened with pre-absorbed serum as detailed previously in this chapter.

Representative blots of the transformants can be seen in Fig 7.4. No novel protein bands were expressed by any of the clones analysed. The bands that were visualised are endogenous *E. coli* proteins, which constitute a relatively weak background binding due to the use of serum pre-absorbed with disintegrated whole cells of *E. coli*.

### **iii) Sequence analysis**

Three representative clones carrying *B. forsythus* DNA of 4-8kb in size were sequenced from both ends using M13 Forward and Reverse primers. Together these reactions yielded sequence information of approximately 1.5kb of the *B. forsythus* sequence. On-line DNA and protein sequence databases were searched for matches with the *B. forsythus* sequence to identify genes that the recombinant clones carried. Two clones carried DNA that appeared to spuriously match genome sequences from other species; as the sequence matches ran for no more than 30 bases these identities were not thought to be significant. The third clone analysed yielded sequence data that shared a high level of homology with sequence from a number of different species, and when the *B. forsythus* inserted DNA sequence was translated to permit a protein sequence search, the clone was found to represent part of the *lac* operon of genes belonging to *B. forsythus*. The ubiquitous nature of the *lac* genes accounts for the homology the recombinant sequence shared with a variety of bacterial species. The sequence data from this clone is displayed in Fig 7.5. An alignment of the putatively identified gene of the *B. forsythus lac* operon with the DNA sequence from another species is shown in Fig 7.6. The sequence alignment was generated using BLAST searches made available by the Institute for Genomic Research through their web-site at <http://www.tigr.org>.



## **DISCUSSION**

With the aim of identifying immunodominant protein antigens of *B. forsythus*, a genomic library was constructed in *E. coli* TOP10, using the plasmid vector pUC18. This is the first report of the construction of a genomic library for *B. forsythus*. As the transformant colonies were exposed to the inducer effects of IPTG, the requisite that protein-coding inserts were expressed with their own promoter was removed.

Of 10,000 colonies screened by colony lift (of which it can be estimated that approximately 1670 carried an inserted gene of the desired size), and 98 subjected to screening via Western blotting, no recombinants were identified that expressed immuno-reactive proteins of *B. forsythus*.

Despite the large number of colonies screened to no apparent avail, the library can not be regarded as a failure statistically until approximately 30,000 clones have been screened. This figure was arrived at using the following equation:

$$P = \text{size of coding region of gene of interest (kb)} / \text{size of genome (kb)} \times 6,$$

where P is the probability of forming a recombinant clone that contains the sequence of the gene of interest in the correct reading frame and orientation for expression. If the average size of the genes that it was aimed were to be expressed in this library can be taken as 6 kb, and the approximate size of the *B. forsythus* genome is 4,500 kb, then  $P=0.00022$ , i.e. there is one clone in 4545 expressing a *B. forsythus* protein. This probability becomes slimmer (1 in 27,270) when the low rate of recombinant carrying inserts, as surmised by restriction endonuclease analysis, is considered. However, as this library was

being screened for any number of different genes that may have been cloned into the library, and not just one particular gene at 6kb, it was anticipated that the chances of detecting a reactive clone would be increased above 1 chance in 27,270. It was therefore thought plausible that a gene of the desired size could have been carried in the 10,000 clones that were analysed.

Around 10,000 clones were screened by the colony lift technique, with no recombinant displaying a significant positive reaction with the patient serum. As it had been determined that this number of clones could contain expressed genes of *B. forsythus*, there was some indication that the expression of *B. forsythus* DNA in *E. coli* TOP10 was poor. This low level of expression of *B. forsythus* proteins may be attributable to differences in synthetic and post-transcriptional pathways that exist between *E. coli* and *B. forsythus*.

Thus a more sensitive assay, Western blotting, was used to detect antigens. Western blotting had previously been shown to detect antigens in a genomic library when whole colony screening was found not to be sensitive enough (Thole *et al*, 1985). Applying this technique to the *B. forsythus* genomic library constructed in this study, 98 recombinant lysates were screened with periodontitis patient serum pre-absorbed with *E. coli* TOP10 lysates. No exogenous reactive proteins were identified using this technique. Analysis of a greater numbers of clones using the colony lift technique, or further Western blots studying recombinant cell lysates, may in time identify an immunoreactive clone. However, the success of both these procedures is entirely limited by the antibodies present in the patient serum sample being used in these studies and if anti-hsp60 antibodies are not present in sufficient numbers no amount of screening will yield a clone or protein band that will

stain positively. Despite the fact that the serum being used in this study had been identified previously as a sample from a periodontitis patient who possessed a strong antibody response to *B. forsythus*, this does not guarantee that the patient carries a high level of antibodies to hsp60. One possible way of circumnavigating this dependency upon one patient serum sample would be to use pooled serum samples which would indubitably increase the antibody repertoire available to recognise *B. forsythus* immunogens. Alternatively, the potential of raising hyper-immune *B. forsythus* sera by repeated immunisation of a laboratory animal should be investigated. Hyper-immune mycobacterial serum was used by Thole *et al* (1985) to screen genomic library Western blots expressing recombinant mycobacterial proteins, but even so, the antigens were reported to stain weakly, close to the detection level, and positive antigen binding was not always reproducible. Thole *et al* attributed the low level of mycobacterial antigen expression to differences in *E. coli* and mycobacterial expression machinery. Nonetheless, altering the serum used for screening would be a desirable step to take when carrying out further work with the *B. forsythus* genomic library.

As no immunogenic clones were identified throughout the screening procedures, it was felt that information may be derived from the genomic library by sequencing the DNA inserted in some of the clones, which is perhaps the ultimate analysis to perform on constituents of a library.

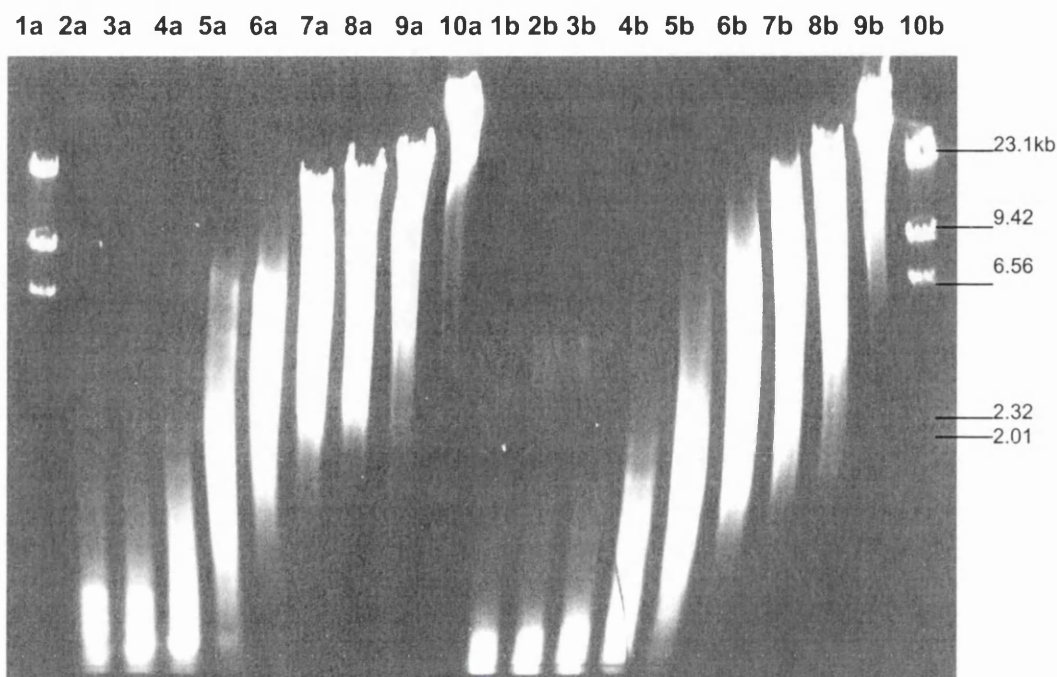
Sequencing random recombinant colonies is also a demonstration of the further analysis that would have been performed on an immunoreactive clone had one been identified, using the same sequencing techniques and database searches that would have been applied to an unknown immunogenic protein of

*B. forsythus*. Preliminary sequence analysis of three recombinant clones from the genomic library, all known to be carrying a DNA insert of 4-8kb, was performed. Approximately 1.5kb of DNA sequence was obtained for each clone, having sequenced each from both ends of the insert. Two of the clones yielded sequence which appeared to only spuriously match sequences contained within SwissProt and EMBL databases, thus it may be speculated that these inserts carry gene sequences that have yet to be characterised. As there was no shared homology with the entire genomic sequence of *E. coli*, these genes may also be species unique. The third clone analysed contained *B. forsythus* DNA sequence that was homologous to 350-base length sequences from a diverse range of prokaryotic species, including *Caulobacter crescentus*, *Enterococcus faecalis* and *Clostridium difficile*. Translated, the same inserted sequence was used to search protein sequence databases, and homology was found to exist with the *lac* operon genes of *E. coli*. It can be assumed that the inserted genes carried by this recombinant clone represent some of the *lac* operon genes of *B. forsythus*. As these genes code for a metabolic system common to many species, the high level of homology this recombinant clone shared with a varied range of eukaryotic species is explained.

It is possible that further screening of this genomic library using patient serum may identify immunoreactive protein antigens of *B. forsythus* as similar procedures have been successfully adopted to identify the protein antigens of *P. gingivalis* (Hayakawa *et al*, 1992). It is possible that there is some amount of digestion of *B. forsythus* proteins by endogenous *E. coli* proteases and that the incorporation of a protease inhibitor at a stage in the procedure may be

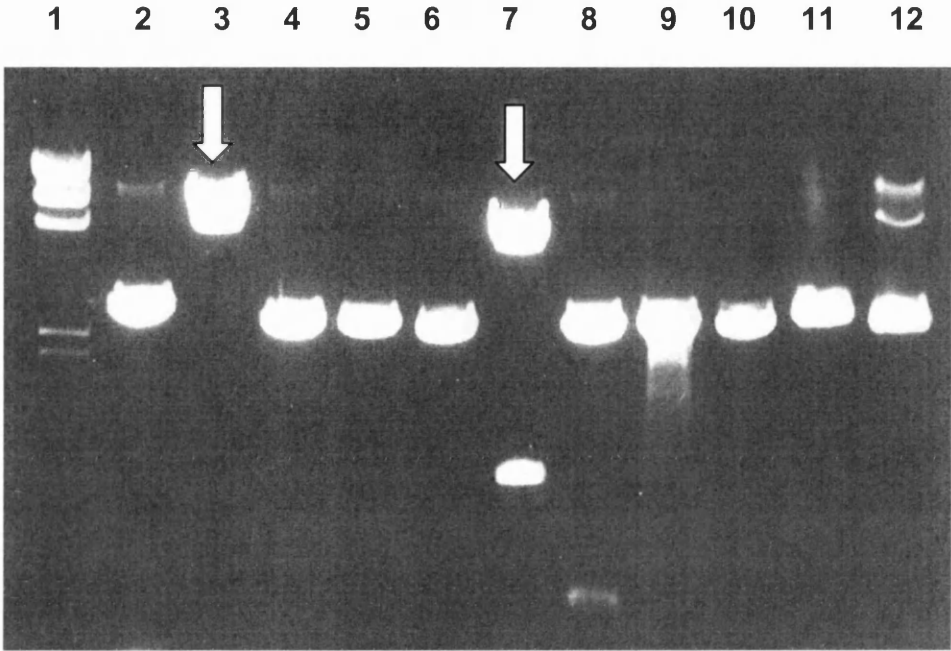
beneficial (Weidner *et al*, 1996). Alternatively, protein specific probes could be designed to search the library. Immunodominant antigens that share sequence homology in other species could be used to design specific PCR primers, and these primers used with a *B. forsythus* template to generate a PCR product that could be labelled to use as a probe as described in Chapter 3. As the vector used in this library is an expression vector, the library could also be screened for recombinant digestion enzyme activity by incorporating a substrate such as casein into SDS-PAGE gels. Cell lysates analysed on these gels that carried a proteolytic enzyme would digest the casein and would be detectable upon silver-staining of the gels.

**FIGURE 7.1. Establishing conditions for partial digestion of *B. forsythus* chromosomal DNA.**



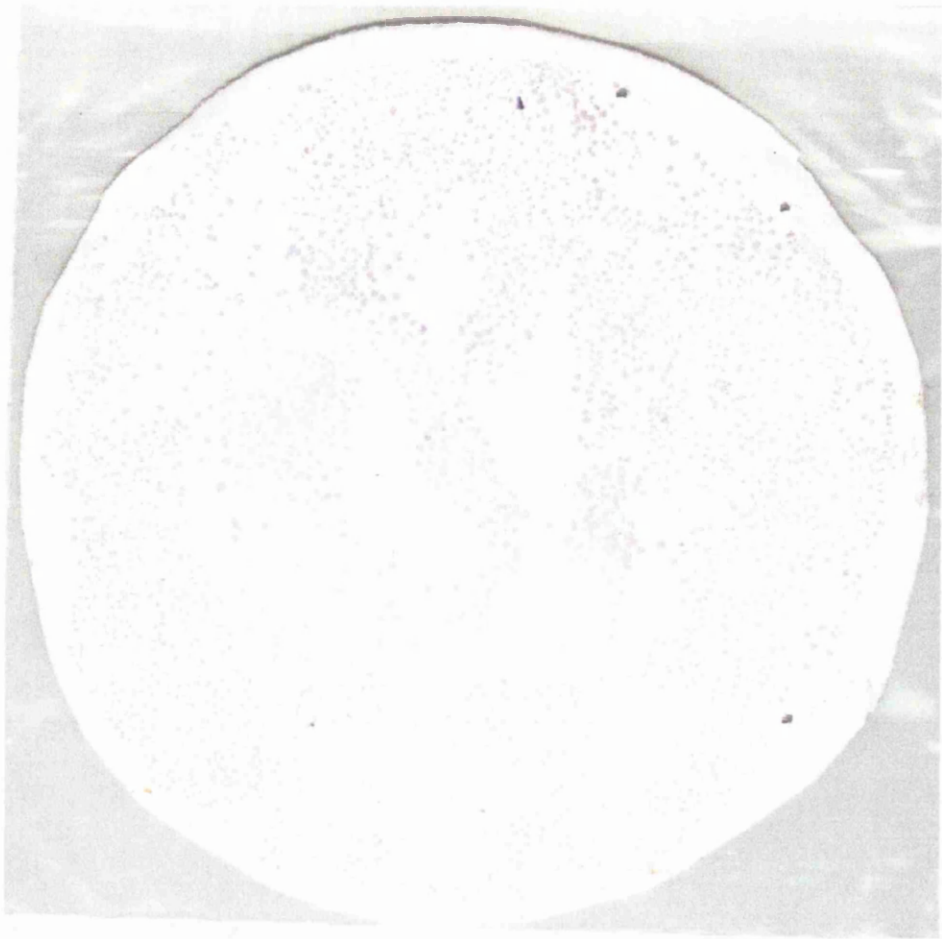
**Figure 7.1.** Establishing conditions for partial digestion of *B. forsythus* chromosomal DNA. Chromosomal DNA was partially cleaved with *Sau3A* to determine optimum conditions for the production of DNA fragments in a size range of 2-10kb. *HindIII* digested  $\lambda$  DNA fragments were used as size markers (lanes 1a and 10b). Decreasing amounts of restriction endonuclease ranging from 2.5U/ $\mu$ g DNA in lanes 2a and 1b to 0.02U/ $\mu$ g DNA in lanes 9a and 8b were incubated with samples and electrophoresed. Lanes 10a and 9b are undigested controls. Gel a) was incubated at 37°C for 1 hour, gel b) for 1 hour 15 min. DNA samples cleaved in lane 6b embrace the optimum conditions, yielding fragments in the correct size range (2-10kb). A subsequent larger scale reaction was performed and cleaved DNA cloned into the vector pUC18.

**FIGURE 7.2.** Mini-prep analysis of recombinant clones from a *B. forsythus* genomic library.



**Figure 7.2.** Mini-prep analysis of recombinant clones from a *B. forsythus* genomic library. Recombinant clones were selected from the library and plasmid DNA purified using the small-scale boiling method. Plasmid DNA was digested with *Eco*RI and released DNA inserts analysed by electrophoresis. *Hind*III digested  $\lambda$  DNA fragments were used as size markers (lane 1; 23.1, 9.42, 6.56, 2.32 and 2.01kb) and digested DNA electrophoresed in lanes 2-12. Recombinant clones carrying a DNA insert in a size range of 2-10kb are seen in lanes 3 and 7 (arrows).

**FIGURE 7.3.** Screening the *B. forsythus* genomic library by the colony lift technique.

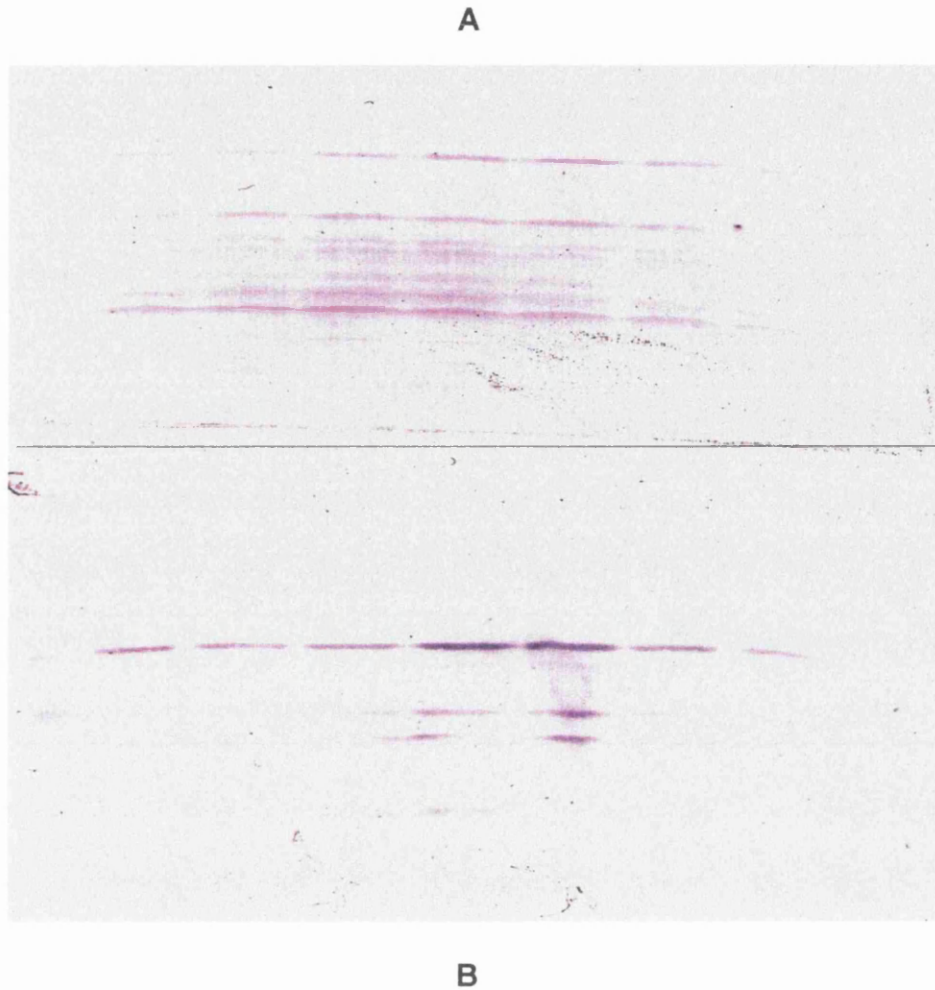


**Figure 7.3.** Screening the *B. forsythus* genomic library by colony lift technique. Samples of the genomic library were grown on nitrocellulose disks on top of L-agar containing IPTG and ampicillin. Replica plates were cultured by imprinting the colonies onto another nitrocellulose disk. The replica colonies were lysed and blotted with serum from a periodontitis patient previously characterised as a high-responder to *B. forsythus*. The serum was pre-absorbed with disrupted *E. coli* whole cells to reduce serum avidity for endogenous host-cell proteins expressed by transformants.

No colonies on the plate illustrated have bound sufficient antibody to indicate expression of a novel immunoreactive *B. forsythus* protein.



**FIGURE 7.4. Screening the *B. forsythus* genomic library by Western blotting.**



**Figure 7.4.** Screening the *B. forsythus* genomic library by Western blotting. Clones from a genomic library were selected and grown in liquid culture in the presence of IPTG and ampicillin. Cells were harvested and lysed prior to SDS-PAGE. Electrophoresed proteins were then Western blotted with serum from a periodontitis patient previously characterised as a high-responder to *B. forsythus*. The serum was pre-absorbed with disrupted *E. coli* whole cells to reduce serum avidity for endogenous host-cell proteins expressed by transformants. 7 samples from the library were analysed per blot (two different blots are shown here, A and B). Expression of a cloned *B. forsythus* gene by a library member would be seen as the production of a novel protein band that was not detectable in other samples. As all samples here display similar protein banding patterns, no representative of the library appears to be expressing *B. forsythus* genes.

## **CHAPTER 8**

### **General discussion**

## **GENERAL DISCUSSION**

Periodontal disease describes a range of conditions that affect the supporting tissues of the teeth, which range from mild inflammation of the gingiva (gingivitis) to advanced periodontitis, in which the deterioration and destruction of tooth attachment ligaments and alveolar bone ultimately results in tooth loss (Kinane, 1997). With the infection and inflammation of the periodontium, a break in the peripheral body defenses is constituted which permits the oral microbial population access to the internal body compartment. As such, periodontal disease may have a significant effect on systemic health, and it has been associated with cardiovascular disease and premature low birth-weight babies (Desvarieux *et al*, 1999; Offenbacher, 1996; Offenbacher *et al*, 1999). With the likelihood that periodontal disease will be linked to further systemic diseases in the future, the need to understand the pathology of periodontitis is now perhaps greater than ever.

A major part of the studies presented in this thesis were concerned with the heat shock protein 60 of the periodontal pathogen *Bacteroides forsythus*. Hsp60 is a molecular chaperone whose function is to aid correct protein folding, assembly and translocation within cells (Craig *et al*, 1993; Ellis and Vandervies, 1991). The importance of hsp60 in cells is reflected by the fact that virtually all cell types are known to express a hsp60 homologue and constitutive expression of hsp60 is essential for cell viability. Synthesis of hsp60 increases upon cell stress, and this is to cope with the increased number of denatured proteins that typically results from cellular stress. Hsp60 is highly immunogenic (Kaufmann, 1990; Kaufmann, 1992a) and therefore is an important antigen, acting as a flag protein for marking invading micro-organisms (stressed as a result of

encountering a foreign environment or immune attack) and infected, traumatised or aberrant host cells alike for immune recognition and subsequent removal. The immune response to hsp60, as a result of constant priming, is rapid and effective. However, it could quickly become a catastrophic auto-immune response if hsp60-reactive T cells and antibodies recruited to a site of local infection or inflammation were to attack healthy host cells expressing hsp60. To prevent this situation from arising, a network of effector mechanisms to control and down-regulate anti-hsp60 responses is thought to exist (Cohen and Young, 1991; Cohen, 1992). The immune system is not infallible though, and the potential failure of it as a result of chronic exposure of the immune system to hsp60 at sites of infection, or as a result of a breakdown in the regulation of anti-hsp60 responses, causes hsp60 to be widely studied as a possible pathogenic agent in a diverse range of infections and diseases; from bacterial infections (Kaufmann, 1991; Kaufmann *et al*, 1991; Shinnick, 1991), inflammatory diseases (Amini *et al*, 1996; Gaston *et al*, 1990; Kaufmann *et al*, 1990; Morrison *et al*, 1989) and cardiovascular disease (Desvarieux *et al*, 1999; Hopplcher *et al*, 1996) to autoimmune disorders (Jones *et al*, 1993; Kaufmann, 1994), neurological disorders (Astarloa *et al*, 1996; Hamos *et al*, 1991; Raine *et al*, 1996) and cancer (Hsu and Hsu, 1998; Ito *et al*, 1998).

Despite the wealth of studies on hsp60, it is only recently that these proteins have become a topic of interest in periodontal research. Various periodontal pathogens are now known to respond to thermal shock (usually inflicted on cells *in vitro* by moving them from a 37°C environment to 42°C for 15 min) by the increased production of hsp60. Oral bacteria shown to respond in this manner include *A. actinomycetemcomitans* (Lokensgard *et al*, 1994; Koga *et al*, 1993),

*P. gingivalis* (Lu and McBride, 1994; Vayssier *et al*, 1994), *T. denticola* (Stamm *et al*, 1991), *B. forsythus*, *P. oralis*, *P. intermedia*, *F. nucleatum*, *Actinomyces viscosus* and *P. buccae* (Vayssier *et al*, 1994). Stresses other than thermal shock, such as pH decrease and increase in oxygen concentration have also been shown to induce the synthesis of hsp60 in oral bacteria (Vayssier *et al*, 1994). Environmental conditions in the periodontal pocket reflect those stresses that have been used in the laboratory to induce hsp60 synthesis in cells: the subgingival temperature is slightly higher at diseased sites as compared to healthy ones, (Kung *et al*, 1990), pH decreases with increasing pocket depth and degree of host inflammatory response (Bickel and Cimasoni, 1985) and phagocytosis of infecting organisms generally exposes the bacteria to a range of reactive oxygen species (Buchmeier and Heffron, 1990). Thus the periodontal pocket is likely to be a site of significantly increased hsp60 expression.

Although hsp60s are typical cytosolic proteins that lack the leader sequence required for transport of the proteins to the cell surface (Kaufmann and Schoel, 1994), it has become apparent that their cellular localisation is not limited to internal cellular compartments, but that hsp60 can also be found on cell surfaces (Jarjour *et al*, 1990; Soltys and Gupta, 1996). Whether this is as a result of an as yet unknown translocation mechanism, or whether cytoplasmic hsp60 is removed to the cell surface via a MHC processing pathway (Jardetzky *et al*, 1991) or is co-translated with an unrelated hsp60-bound protein (Kaufmann and Schoel, 1994) is unclear. What is increasingly clear though is that these proteins are widely available for recognition by the host immune response.

With regards to the hsp60s of periodontal pathogens, characterisation of patient immune responses to these proteins has followed on from studies that have cloned and sequenced the hsp60 genes of the major periodontal pathogens *A. actinomycetemcomitans* and *P. gingivalis* (Nakano *et al*, 1995 and Maeda *et al*, 1994 respectively). Antibodies recognising hsp60s from oral bacteria have been shown to be widely cross-reactive (Ando *et al*, 1995; Hinode *et al*, 1998), although it is important to stress that the level of cross-reactivity is entirely dependent on the epitope to which the antibodies were raised. Certainly Nakano's study (1995) illustrates that monoclonal antibodies raised against the 64kDa antigen of *A. actinomycetemcomitans* did not react with any cellular proteins from a range of oral bacteria. Also, the level of cross-reactivity an epitope may stimulate cannot be estimated by the level of sequence identity which that epitope shares with other homologues alone (Yi *et al*, 1993). That the antibody response to hsp60 may be of significance in the periodontal disease process has been suggested by studies that demonstrate that the level of hsp60 reactive antibodies in the serum of periodontitis patients is higher than it is in healthy controls (Tabeta *et al*, 1999; Schett *et al*, 1997; Ando *et al*, 1995; Maeda *et al*, 1994). Samples taken from patients with adult periodontitis and rapidly progressive periodontitis have also been seen to possess antibodies reactive with the human homologue of hsp60 (Tabeta *et al*, 1999; Ando *et al*, 1995), and have initially been taken to indicate the occurrence of an autoimmune response. Hsp60 reactive antibodies may perform as components of disease processes in a number of ways. Firstly, as hsp60 has been shown to reside on the surface of cells, hsp60 antibodies may have a direct cytotoxic effect, and could help maintain inflammatory processes by perpetuating a high level of host

cell death. Secondly, hsp60 antibodies bound to hsp60 molecules may impede the proteins' ability to function, and thus render cells more susceptible to death due to the cell's inability to cope with changes in the immediate surrounding environment (Riabowol *et al*, 1988). Lastly, several authors now share the opinion that the demonstration of healthy serum samples carrying antibodies to hsp60 can be explained by the supposition that hsp60 antigens are presented to individuals constantly, to prime the immune system to respond to infecting organisms and aberrant host cells efficiently and to ensure that a level of regulation of the immune response to hsp60 is constantly maintained (Cohen 1992; Cohen and Young, 1991; Kaufmann, 1994).

As hsp60 is a ubiquitous and constitutively expressed antigen, it is evident that a level of control of the immune response to this protein is fundamental, otherwise the host would rapidly become involved in a programme of autoimmune self-destruction upon initiation of any infection or inflammatory process. Homeostatic regulation of the immune response to hsp60 would presumably be achieved by suppressor T-cells specific for hsp60, or anti-idiotypic T-cells, whose sustainment would be dependant on hsp60 presentation to T-cells, possibly via the MHC gene products (Jardetzky *et al*, 1991). Elevated levels of antibodies against hsp60 could feasibly interfere with this presentation of hsp60 by MHC to control elements of the immune response. As a result, hsp60 antibodies would disrupt the homeostasis, and autoimmune disease could ensue. If this last theory was applied to explain the autoimmune component of periodontitis, the leading question ensuing from this idea is why is periodontitis, like other autoimmune diseases in which reactivity to hsp60 has been implicated, a localised disease? Surely the collapse of a homeostatic

network that down-regulates the immune response to hsp60 would result in the cataclysmic self-destruction that the regulatory pathways strive to defend against? It may be that the autoimmune reaction is directed against a localised tissue-specific hsp60 antigen (Jones *et al*, 1993), which would presumably only be expressing high levels of hsp60 at the site of inflammation.

The data supporting or disproving these theories has yet to materialise, as the studies on hsps in periodontitis to date have only examined small patient groups of around 20 subjects. Whilst the idea of hsp60 being involved in autoimmune process of periodontitis is an attractive one, seemingly supported by the finding of human-hsp60 reactive antibodies (Ando *et al*, 1995; Tabeta *et al*, 1999), it should be borne in mind that the identification of self-reactive anti-hsp60 antibodies need not signify autoimmune disease. Rather, these may be normal, healthy autoimmune reactions in which hsp60 production has marked a stressed host cell for immune surveillance. This notion of an immune surveillance system primed to rapidly respond to hsp60 'flags' is supported by findings that significant proportions of  $\gamma/\delta$  T-cells, thought to have a function as surveillant cells of the immune system due to their extra thymic development and ability to recognise only a limited antigenic repertoire (Haas *et al*, 1993; Haas and Tonegawa, 1992), have been shown to recognise hsp60 (Fisch *et al*, 1990; Haregewoin *et al*, 1989; Kaufmann and Kabelitz, 1991).

Hsp60 molecules of some species are considered to be virulence factors. As such, the hsp60 molecules could be implicated more directly with the infective state of diseases. Buchmeier and Heffron (1990) demonstrated that hsp60 induced in *S. typhimurium* was able to stabilise bacterial macromolecular complexes after they had been exposed to toxic and degradative products upon



digestion by macrophages, indicating that the resistance of *S. typhimurium* to phagocytic killing is attributable to the increased production of bacterial hsp60 upon phagocytosis. The 60-kDa hsp of *S. typhimurium* has also been shown to be responsible for the binding of the bacterium to intestinal mucus (Ensgraber and Loos, 1992).

Determining the means by which hsp60 of oral pathogens contributes to disease mechanisms of periodontitis, if at all, is beyond the scope of current research results in this field. Even if it should transpire that hsp60 is not the harbinger of disease that some currently think it is, benefits can still be drawn from the work already done on hsp60s in periodontitis. For example, Schett *et al* (1997) have suggested that levels of hsp60 antibodies present in the saliva and serum of patients could be used as a diagnostic aid for gingivitis and periodontitis. Hsp60 antibody titres have been widely proposed as diagnostic tools to mark patient sub-populations within different groups (Gruber *et al*, 1996; Portig *et al*, 1997; Prakken *et al*, 1996; Peeling *et al*, 1997). Secondly, hsp60 of bacterial origin are a possible target for vaccine development against periodontitis.

The development of a vaccine effective against periodontitis is perhaps, to the minds of many, the ultimate goal of research ongoing in this field of dentistry today. Many argue that periodontitis is frequently a self-inflicted result of poor dental hygiene in the individual, and adhere to the doctrine that prevention is better than cure, and can easily be attained with improved standards of oral care. However, the need to provide a vaccine against infections of the periodontium is a very real one, for a number of reasons.

It is now apparent that individuals possess genetic factors that may pre-dispose them to periodontal disease (Genco, 1996). Evidence exists which relates human leukocyte antigen phenotype distribution with the prevalence of periodontal disease types (Amer *et al*, 1988; Reinholdt *et al*, 1977). Localised juvenile periodontitis is suggested to be a familial disease, inherited via an autosomal mode of transmission, (Hart *et al*, 1992) which causes an abnormality in neutrophil function (Van Dyke *et al*, 1985). As genetic control plays an important role in many instances of periodontitis, the development of a vaccine to treat disease would be a vital tool to treat those of an afflicted lineage, for whom periodontal disease might otherwise be an inevitable consequence.

Oral diseases are being widely investigated now as a source of bacterial infection that may incur systemic disease at sites distal to the oral cavity in an affected host. In particular, recent studies have found an association between periodontal disease and cardiovascular disease, including myocardial infarction (Mattila *et al*, 1989; Wu *et al*, 1999), ischemic stroke (Young *et al*, 1999; Veber *et al*, 1999) and atherosclerosis (Matilla *et al*, 1993; De Stefano *et al*, 1993; Schett *et al*, 1997; Desvarieux *et al*, 1999). Of interest is the finding that *B. forsythus* specifically, along with *P. gingivalis*, is associated with an increased risk for myocardial infarction (Wu *et al*, 1999). Hsp molecules too have been specifically implicated: elevated serum antibody levels to hsp60 have been correlated with a high incidence of carotid and coronary atherosclerosis (Hopplicher *et al*, 1996). These antibodies may be cytotoxic to stressed human endothelial cells, such that antibodies induced by oral infection may be a cause of endothelial cell damage. This would suggest that dental infection may be a

condition that favours the development of cardiovascular disease, and as one of the most significant causes of mortality in the Western world, the ability to vaccinate against a contributing factor to heart disease would have unequivocal benefits.

Lastly, well-publicised current opinion holds that the antibiotic era in which the 20th century population has thrived is on the wane. Antibiotic resistance in *B. forsythus* has already been recorded (Maiden *et al*, 1994; van Winkelhoff *et al*, 1997). This necessitates the development of a new generation of drugs to combat infection, and it is likely that these will be sophisticated modulators of our own immune response that will adapt our available immune components to target specific infectious agents. These are, in short, vaccines. Traditionally vaccines originally consisted of killed whole organisms, or serum factors raised against an infectious agent in an animal host. Formalin killed whole *P. gingivalis* has been used as a vaccine which was protective against ligature-induced periodontitis in non-human primates (Persson *et al*, 1994). However, whole Gram-negative bacteria are considered to be unsuitable human vaccine components due to the risk of possible side effects (Ishikawa *et al*, 1997) which can counter balance the benefits of this vaccine and others developed in a similar manner. However, the advent of modern techniques that can create synthetic non-harmful components of antigenic organisms, and the use of human cell lines to culture immune factors, will expand the technology of vaccine development immensely, and the problems faced in this science 20 years ago can now be largely overcome.

The idea that immunisation may be an effective means to prevent and control periodontal disease has been bolstered by findings that show that patients with

high serum antibody titres to suspected periodontal pathogens have less severe disease than patients with low titres of the same antibodies (Ranney *et al*, 1992; Gunsolley *et al*, 1987). High titre sera has been demonstrated *in vivo* to opsonise and enhance killing of periodontal pathogens by polymorphonuclear leukocytes in the presence of complement (Underwood *et al*, 1993). However, periodontitis offers particular problems that hinder the production of a successful vaccine. Firstly, periodontal disease is not attributable to any one organism, but is an infection by a mixed population of bacteria, the majority of which are Gram-negative anaerobes (Moore and Moore, 1994). Although this group has been well defined and patterns of multiple infections have been characterised for different disease groups (Haffajee *et al*, 1988a; Haffajee *et al*, 1988b; Haffajee and Socransky, 1994) it remains that no two infections are identical, and microbial populations vary from host to host, and even from site to site in the same host. Thus the elimination of no single target organism could guarantee the eradication of disease. Furthermore, a significant amount of variation in strains within species makes for difficulties when selecting a vaccine target. A recent study has shown that at least 10 genetically distinct forms of *B. forsythus* exist (Takemoto *et al*, 1997), illustrating the potential for phenotypic variation.

The development of a successful vaccine therefore requires the identification of strain variable components, so that vaccines will not be targeted against these antigens which cannot offer broad-spectrum protection against infection.

Similarly, the identification of antigens shared amongst different species could allow immunisation by one species that could induce cross-reactive antibodies that may confer protection against infection by other Gram-negative species.

With the aim of identifying antigenic components of *B. forsythus* clinical isolates, Sims *et al* (1998a) screened 10 isolates with the sera from 10 patients with early onset rapidly progressive periodontitis. Their study showed the dominant strain-variable components of *B. forsythus* were non-protein, whilst high molecular weight proteins (200 and 210kDa) were the major cross-reactive components. Sims suggests that both these antigenic factors are associated with the surface layer (S-layer) of *B. forsythus*, with the proteinase-resistant non conserved material possibly being oligosaccharide released from S-layer proteins (similar structures in other organisms are known to be glycosylated) and the conserved proteinaceous immunogen perhaps being a structural sub-unit of the S-layer. The S-layer of *B. forsythus* is a crystalline protein or glycoprotein structure, consisting of serrated subunits forming a tetragonal pattern, that lies outside the outer membrane (Kerosuo, 1988). S-layer proteins may be serologically important in *B. forsythus* as the constituents are amongst the most abundant proteins expressed by the organism, and form the outer-most surface of the bacterium. The S-layer proteins of *B. forsythus* have been shown to be immunogenic by a pre-absorption study in which sera was pre-absorbed with purified S-layer proteins, and a corresponding drop in antibody titre, of as much as 98%, was observed (Sims *et al* 1994).

Another study suggests that the lipopolysaccharide (LPS) of *B. forsythus* may be a good target antigen for a periodontitis vaccine. An antigen was shown to be cross-reactive with *P. gingivalis* and *B. forsythus* by immunising monkeys with *P. gingivalis*, and then demonstrating that the antibodies raised against *P. gingivalis* recognised 10 different *B. forsythus* isolates (Vasel *et al*, 1996).

These potentially protective antibodies bound epitopes in lipid A and possibly in

the core carbohydrate of LPS. Again this antigen is non-protein, and it is important to note that screening for immunodominant antigens expressed in a genomic library, as was performed in this thesis (Chapter 7) precludes serum reactivity with carbohydrate antigens, which may be the truly dominant antigens. A pertinent observation recorded in the results of this study was that different individuals afflicted with the same condition (ie. adult periodontitis) possess varied antibody responses. Of 11 serum samples collected from rapidly progressive periodontitis patients, 3 displayed a strong antibody reactivity to *B. forsythus* hsp60. That the other patients did not display reactivity with *B. forsythus* hsp60 does not necessarily mean that these patients were not infected with *B. forsythus*, in fact recent data would suggest that these patients are likely to be carrying the organism. Rather, it indicates that each individual's ability to respond to antigens is unique, and reflects the random manner in which the antigen recognition repertoire is formed. A similar finding was reported by Sims *et al* (1998a; 1998b). Also performing a study with serum from patients with rapidly progressive periodontitis, serum samples were screened against 10 different *B. forsythus* clinical isolates, and a widely divergent IgG binding profile was demonstrated amongst the patients. One serum sample would stain heavily for IgG binding to an isolate that another serum sample only weakly recognised. Thus patients seem to be infected by, and respond to, different *B. forsythus* serotypes.

Their universal conservation and cross-reactivity makes hsp60 molecules attractive candidates for the development of vaccines against various diseases. However, the very features that make hsp60 proteins promising vaccine aspirants may pose problems with regards to vaccine target specificity, and

ultimately rule-out the effective beneficial use of vaccines against hsp60.

Furthermore, it has been suggested that increased antibody titres to hsp60 antigens may play an intrinsic part in disease pathogenesis, and the antibody response has been directly implicated in causing disease. Clearly a vaccine that raised these very same antibody titres would have an adverse effect on the individual.

The data presented in this thesis reports the identification and DNA sequence determination of the hsp60 gene of *B. forsythus*, and the partial sequence of the hsp60 gene of *P. intermedia*. Analysis of the predicted amino acid sequences of these proteins and comparison with available sequences of hsp60 homologues from other species demonstrated that the proteins possess highly conserved primary and predicted secondary structures. Further to this, recombinant *B. forsythus* hsp60 was synthesised in *E. coli* and the immunoreactivity of this protein assessed by screening healthy and chronic adult periodontitis patient serum samples for the presence of reactive antibodies. A low frequency of samples from both groups recognised *B. forsythus* hsp60, indicating that the protein is immunogenic, but probably not pathogenic. The results of the immunological analysis of *B. forsythus* hsp60 presented here are comparable with studies on hsp60 from other oral pathogens. Maeda *et al* (1994) reported antibodies reactive with recombinant *P. gingivalis* hsp60 in periodontitis and healthy patient serum samples, and antibodies reactive with hsp60 of *A. actinomycetemcomitans*, *F. nucleatum* and *P. nigrescens* have also been recorded in periodontitis serum (Ando *et al*, 1995). Two other reports differ in that no hsp60 antibody reactivity to *A. actinomycetemcomitans* or *P. gingivalis* hsp60 is reported in healthy serum samples, although it is detected in

periodontitis patient samples (Koga *et al*, 1993; Tabeta *et al*, 1999).

Interpretation of any of these results, which essentially represent preliminary reports, is limited by the small size of patient sample numbers analysed. For larger numbers of samples to be studied, it would be of significant benefit to procure a protocol for purifying recombinant *B. forsythus* hsp60, and this would be the most desirable 'next-step' to follow work done on this thesis.

Whether the immune response against hsp60s is protective, destructive or inconsequential to the progression of disease is unknown. On the one hand, it has been suggested that high levels of hsp60 antibodies are indicative of disease (Peeling *et al*, 1997; Prakken *et al*, 1996; Schett *et al*, 1997) whilst in direct contradiction to this, low levels of hsp60 antibodies has also been suggested to indicate disease as a result of ineffective immune recognition of micro-organisms (NEW). Neither is it evident why a higher proportion of patients do not recognise hsp60 antigens, as they have been consistently illustrated as extremely immunogenic, and are the dominant protein expressed by stressed cells. With the nature of our immune response to hsp60 apparently so ambiguous, much work remains to be done if we are to fully understand the nature of these evolutionarily ancient proteins and the significance of our immune response to them.



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